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1. Your reference C2550-GB

2. Patent application number *(The Patent Office will fill in this part)* 0319118.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 8693285001

If the applicant is a corporate body, give the country/state of its incorporation BE

4. Title of the invention Variable antibodies

5. Name of your agent *(if you have one)* William E. Bird of Bird Goën & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Bird Goën & Co
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London NW1 8BB
United Kingdom

Patents ADP number *(if you know it)* 8675910001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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William E. Bird 14 August 2003

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VARIABLE ANTIBODIES

FIELD OF THE INVENTION

The present invention relates to the modification of inhibitory antibodies in order to achieve a variable maximal inhibitory activity and its application in the development of antithrombotic agents.

BACKGROUND OF THE INVENTION

The formation of blood clots does not only limit bleeding in case of injury (haemostasis) but can occlude important arteries or veins, leading to serious organ damage and death. Thrombosis is thus blood clot formation at the wrong time and place.

Upon damage of a vessel, the coagulation (clotting) system is immediately initiated producing thrombin and blood platelets adhering to matrix proteins, which in turn leads to the aggregation of additional platelets into a growing platelet plug in concert with the conversion of fibrinogen in the blood to the insoluble fibrin.

At each step of the coagulation cycle, a clotting factor zymogen undergoes limited proteolysis and itself becomes an active protease. This clotting-factor enzyme activates the next clotting factor zymogen until thrombin is formed which connects fibrinogen to the insoluble fibrin clot. The blood clotting factors include factor I (f ibrinogen) , factor II (prothrombin) , tissue factor (formerly known as factor III) , factor IV (Ca 2+), factor V (labile factors), factor VII (proconvertin), factor VIII (antihemophilic globulin, or 11AHG11), factor IX (Christmas factor), factor X (Stuart factor), factor XI (plasma thromboplastin antecedent, or "PTA"), factor XII (Hageman factor), factor XIII (fibrinstabilizing factor), and factors HMWK (high-molecularweight kininogen, or Fitzgerald factor), PREK (prekallikrein, or Fletcher factor), Ka (kallikrein), and PL (phospholipid).

Fibrinogen is a substrate for the enzyme, thrombin (factor IIa), a protease that is formed during the coagulation process by the activation of a circulating zymogen, prothrombin (factor II). Prothrombin is converted to the active enzyme thrombin by activated factor X

in the presence of activated factor V, Ca 2+ and phospholipid. Two separate pathways, called the "intrinsic" and "extrinsic" systems, lead to the formation of activated factor X. In the intrinsic system, all the protein factors necessary for coagulation are present in the circulating blood. In the extrinsic system, tissue factor, which is not present in the circulating blood, is expressed on damaged endothelium, by activated monocytes, by cells in the arteriosclerotic plaque or by cells outside the vessel wall. Tissue factor then acts as the receptor and essential cofactor for the binding of factor VII, resulting in a bimolecular enzyme (tissue factorVIIa) to initiate the extrinsic pathway of coagulation. This mechanism also activates the intrinsic pathway of coagulation.
As a summary, the coagulation system involves a cascade of complex and regulated biochemical reactions between circulating blood proteins (coagulation factors), blood cells (in particular platelets) and elements of an injured vessel wall.

Venous thromboembolic disease (deep vein thrombosis, pulmonary embolism, atrial fibrillation) remains a major health issue, with an incidence of 1 to 3 per 1000 individuals per year and a high early mortality rate (Nordstrom et al., 1992; Rosendaal et al., 1997).

Current anticoagulant therapies primarily consist of heparin (or low molecular weight heparins) and vitamin K antagonists, which are both unsatisfactory and inconvenient. All treatments carry a significant risk of bleeding (Research Committee of the British Thoracic Society, 1992), which limits both the dose and duration of treatment and may require regular monitoring (Hylek et al., 1994, Cannegieter et al., 1995). New drugs are currently being developed, but none appears to match optimal criteria of efficacy, safety and convenience.

Antibodies directed to coagulation factors were recently developed as anticoagulant agents . Antibodies directed against Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, factor XIa, Factor VIII, Factor VIIIa, Factor V, Factor Va, Factor VII, Factor VIIa, thrombin, the Von Willebrand Factor, Tissue Factor and other elements of the coagulation cycle have already been described.

WO 97/26010 discloses antibodies inhibiting coagulation in what is described as "a self-limited manner". These antibodies are characterized by the fact that high concentrations of such antibodies prolong coagulation tests such as the APTT only in a limited manner and will not render blood unclottable in contrast to high doses of anticoagulant agents such as heparin. However, a limited increase in APTT does not exclude the risk of bleeding. It is not clear whether these antibodies having so-called "self-limiting neutralizing activity" can avoid completely neutralizing their target coagulation factor, thereby exposing the patient to high bleeding risks. Indeed, in patients with complete deficiency of coagulation factors such as FVIII or FIX, APTT is also prolonged in only a finite manner. The blood of such patients is also not uncoagulable in contrast to blood treated with high doses heparin. However, such patients with severe FVIII or FIX deficiency suffer from dramatic hemorrhagic diseases called hemophilia A or B. As antibodies inhibiting coagulation factors in a "self-limited manner" have biological activities mimicking the blood defect in these patients, they may expose the patients to high bleeding risks.

WO 01/04269 discloses a human monoclonal antibody, Krix-1, which only partially inhibits FVIII activity whatever the excess of antibody over FVIII. This limited inactivation of FVIII was called a "plateau effect". By comparison with antibodies having "self-limiting neutralizing activity", antibodies such as Krix-1 have the advantage that they cannot completely inactivate the target coagulation factor. WO 01/04269 A1 discloses that despite this limited FVIII inactivation, Krix-1 was efficient in preventing thrombosis in a hamster model of venous thrombosis. This antibody was also effective in a mouse model of vena cava thrombosis (Singh et al., Blood 2002). Krix-1 inhibits about 90% FVIII activity (range 85-95%) in normal human plasma.

Factor FVIII therefore appears as a potential target for anticoagulant drugs. However, it is likely that the bleeding tendency associated to the use of anti-FVIII antibodies will be related to the degree of inhibition of the target coagulation factor. It is therefore important to establish methods to generate antibody preparations with an optimal ratio between efficacy (antithrombotic action) and safety (low bleeding tendency).

So far, all of the anticoagulant agents tested in clinical studies are associated with an important risk of bleeding. Besides, LMWH requires frequent subcutaneous administrations and coumarin derivatives require regular monitoring.
Safer and more efficient methods for the prevention and treatment of venous thromboembolic diseases are therefore desirable. Ideal anticoagulant agents should not carry a risk of bleeding complications or of overdosing. They should not require regular monitoring, be easy to administer and well-tolerated. Finally, an antidote should be available.

As a summary, there is still a stringent need for good anti-coagulant therapies with better safety/efficacy ratios.

SUMMARY OF THE INVENTION

The present invention relates to a method for modifying the inhibitory activity of an antibody, without significantly affecting the affinity. The invention further relates to antibodies obtained by such a method and their use in developing antibody mixtures with a variable maximal inhibitory activity of their protein target. More in particular, this invention relates to human monoclonal antibodies that can be modified to partially inhibit in a variable maximal manner a coagulation factor and are used as antithrombotic agents.

In the present invention, a method is provided for modifying the inhibitory activity of an inhibitory antibody by modification of the glycosylation in a variable region of the antibody, whereby the affinity of said antibodies for their target protein affected in a limited way. Particularly, according to one aspect of the present invention the dissociation konstant of the modified antibody is modified by a factor less than 3, preferably less than 2, most preferably less than 1.5. The present invention demonstrates that antibodies or fragments thereof can be developed with a modified inhibitory capacity, but an similar affinity, by modification of the glycosylation in a variable region of the antibody. More particularly, the present invention relates to a method for

decreasing the inhibitory activity of an inhibitory antibody by deglycosylation. These antibodies are of use in situations where a variable or sub-maximal inhibition of a target protein is required, as in the field of coagulation.

The current limitation of human monoclonal antibodies to FVIII is that there is no method allowing the production of antibodies with any given "plateau inhibition", which would allow the selection of the antibodies with an optimal ratio between safety and efficacy to treat or prevent thrombosis.

A further aspect of the present invention relates to a method of obtaining antibodies with variable maximal inhibitory activity but similar affinity by modifying the glycosylation in the variable region. This method is particularly suited for those antibodies where the epitope of the target protein corresponding to antigen-binding site is in the vicinity but does not correspond exactly to the active or interactive site of said protein. The modification of glycosylation is optionally obtained by exposing the native antibodies to carbohydrate cleaving or transforming enzymes. Alternatively, the antibodies with modified glycosylation are obtained by producing the antibodies in cell lines with suitable glycosylation enzymes or by modifying the cell culture conditions to modify the activity of the glycosylation enzymes of the cell line producing the antibodies. In another embodiment of the present invention, the antibodies with modified glycosylation are obtained by genetically modifying the antibody in order to remove or introduce glycosylation sites, for example by site-directed mutagenesis. Antibodies with a modified inhibitory capacity and an substantially unaffected affinity, are optionally identified by measuring the inhibitory capacity and the affinity of a native antibody, modifying the glycosylation of the antibody and again measuring the inhibitory capacity and the affinity of the modified antibody.

The present invention further relates to inhibitory antibodies obtained by the method of the invention, with modified glycosylation and a modified inhibitory activity, characterised in that the affinity of said antibodies for their target protein is substantially unaffected. The invention also relates to fragments, derivatives and homologs of said

antibodies. The antibodies of the present invention include fragments thereof such as, but not limited to, Fab fragments or F(ab')2 fragments.

The present invention also relates to mixtures of said antibodies or mixtures of the antibodies of the invention with other antibodies, like for example a mixture of antibodies having modified glycosylation with the native unmodified antibody whereby variable maximal inhibitory capacities are obtained for said mixtures.

According to one aspect of the present invention, the method of the invention is applied in the development of antibodies directed against a protein which is involved in a complex, i.e. a protein which for its biological function, requires interaction with other proteins. In a more particular embodiment of the present invention, the antibodies of the invention are directed against an element of the haemostasis system or to polypeptides or other molecules which bind to an element of the haemostasis system, yet more in particular to factors of the coagulation cascade, in order to obtain antibodies with variable inhibitory effect on blood coagulation. Thus, the present invention relates to inhibitory antibodies directed against Factor V, Factor VII, Factor VIII (FVIII), Factor IX, Factor X, Factor XI, thrombin, the Von Willebrand Factor or other elements of the coagulation cascade, modified to obtain variable maximal inhibitory activity.

In a more particular embodiment of the invention, antibodies demonstrating variable maximal inhibition of FVIII are disclosed. In a yet more particular embodiment, these antibodies have a FVIII inhibitory capacity between 20% and 90%, more in particular between 30% and 80%, yet more in particular between 40% and 70% and still more in particular between 50% and 60%. A particular embodiment of the invention is a mixture of two or more inhibitory antibodies against FVIII, to achieve a given maximal inhibition of FVIII whatever the excess of the mixture of antibodies over FVIII.

The present invention further relates to anticoagulant monoclonal antibodies derived from Krix-1 and with altered glycosylation resulting in a variable maximal partial inhibition of FVIII activity. More particularly the invention relates to an anticoagulation factor

monoclonal antibody inhibiting less than 65% of FVIII activity and preventing thrombosis in mammal models of thrombosis

The present invention further relates to anticoagulant antibodies directed against FVIII derived from a cell line called Krix-I, with a modified inhibitory activity but retaining similar affinity for FVIII as Krix-1. In a more particular embodiment said antibody is derived from Krix-1 or a fragment thereof or a recombinant produced analogue of such a modified antibody, more in particular the variable regions of said antibody have an amino acid similarity of at least 80%, preferably at least 90% or more with Krix-1 or a fragment thereof. Such antibodies include antibodies comprising an immunoglobulin heavy chain variable region comprising a sequence having at least 80%, prefarably at elast 90%, more preferably at least 95%, most preferably at least 98% sequence homology with SEQ ID NO:1, wherein Asn at position 47 has been modified to Glutamine, Aspartic acid, or Glutamic acid or wherein Thr at position 49 has been modified to Alanine.

Such antibodies include antibodies obtained by chain shuffling whereby the antigen binding site of the antibody has the epitope specificity of the modified Krix-1, e.g. has the epitope specificity of LE2E9Q or LE2E9A. Such antibodies further include fragments of the modified Krix-1 or fragments of Krix-1 modified according to the present invention, provided they have anti-coagulant activity. Thus, the invention also relates to a chimeric antibody comprising a heavy and a light chain, wherein a variable region of said antibody is modified to introduce or remove N-glycosylation site, said antibody characterized by inhibiting the function of a coagulation factor in a limited manner, whereby thrombosis is inhibited and partial inhibition of coagulation is achieved.

In a particular embodiment of the present invention, said antibody is the recombinant antibody of Krix-1 or a fragment thereof, produced in any suitable host cell, e.g. in CHO cells. In a yet more particular embodiment, said antibody is a mutant of Krix-1 with modified N-glysosylation in the variable region, more particularly with a mutated glycosylation site at positions Asn47 to Thr49, more in particular with Asn47 changed to

Gln47 (LE2E9Q), Glu47 (LE2E9E) or Asp47 (LE2E9D) and/or Thr49 to Ala49 (LE2E9A).

Moreover, the invention relates to the cell lines producing the antibodies according to the present invention, more particularly the cell lines producing antibodies with altered post-translational modifications, more particularly with the characteristics of Krix-1, LE2E9Q or LE2E9A.

The invention further relates to the use of said antibodies, mixtures of said antibodies and mixtures of said antibodies with the corresponding unmodified antibodies for the controlled inhibition of biological processes. Thus another aspect of the invention is a pharmaceutical composition comprising one or more antibodies of the invention and a pharmaceutically acceptable carrier.

More particularly, the usefulness of antibodies with modified inhibitory activity is demonstrated in the field of coagulation disorders. The antibodies according to the invention are of use for the controlled inhibition of coagulation. Thus the invention relates to the use of antibodies with modified inhibitory activity of coagulation factors in the manufacture of a medicament useful for the treatment of subjects suffering from a coagulation disorder, more in particular from a venous thromboembolic disease. The present invention furthermore relates to a method of treatment of coagulation disorders by using said antibodies. A particular object of the present invention is to provide an effective anti-thrombotic therapy with reduced risk of bleeding in animals, particularly in humans. This is achieved with the use of the antibodies of the present invention with modified maximal inhibitory activity of coagulation factors and mixtures thereof, more particularly with the use of inhibitory antibodies directed against FVIII with modified maximal inhibitory activity.

Accordingly, one aspect of the present invention is a method of treatment comprising administering an effective dose of one or more therapeutic monoclonal antibody(ies) or fragment(s) thereof modified in such a way as to modify or introduce a glycosylation site in the antibody in order to modify the inhibitory effect of the said antibody on the

activity and/or the interaction(s) of the ligand recognized by the said antibody(ies) with other proteins or reagents interacting with the said ligand.

Particularly, according to the present invention, a method is provided for inhibiting thrombosis comprising administering an effective dose of one or more monoclonal antibody or fragment(s) thereof which inhibit(s) a factor involved in coagulation, modified in such a way as to modify or introduce a glycosylation site in the antibody in order to modify the inhibitory effect of the said antibody on the interaction(s) of the ligand recognized by the said antibody with other proteins or reagents interacting with the said ligand. A specific embodiment of the present invention is a method for adjusting antithrombotic treatment to the clinical situation of the patient based on varying the maximal inhibitory activity of anti-coagulating antibodies. Thus, the present invention relates to the formulation of a medicament for the treatment or prevention of thrombosis, taking into account the clinical situation of the patient, which comprises the selection of one or more antibodies in order to obtain maximal inhibitory activity, suitable for said treatment.

Accordingly, one aspect of the present invention is pharmaceutical formulation for inhibiting thrombosis, which can be adjusted based on the clinical needs of the patient, comprising an effective dose of an anticoagulant monoclonal antibody or a mixture thereof derived from Krix-1 in which the glycosylation in the variable region has been modified, optionally in a mixture with unmodified Krix-1. . More particularly the pharmaceutical compound comprises one or more monoclonal antibodies which have been modified in the glycosylation located at Asn47-Thr49. Optionally this modification is a mutation, more in particular with Asn47 changed in Gln47 (LE2E9Q), Glu47 (LE2E9E) or Asp47 (LE2E9D) and/or Thr49 in Ala49 (LE2E9A). Alternatively, this modification is obtained by contacting the native Krix-1 antibody or fragments thereof with conditions which ensure modification of the glycosylation at Asn47-49.

Accordingly, one aspect of the present invention is a library comprising at least two anticoagulant antibodies with variable maximal inhibitory capacity of FVIII but with

similar affinity for FVIII. According to a particular embodiment said library comprises antibodies derived from Krix-1 by modulation of the glycosylation of the variable region of Krix-1. The invention further relates to a method for manufacturing a medicament for the controlled treatment or inhibition of coagulation in the treatment of thrombosis, comprising selecting one or more of said anticoagulant monoclonal antibody(ies) derived from Krix-1 from the library of the invention. Particularly, the library comprises antibodies derived from Krix-1 by deglycosylation of the glycosylation site located at Asn47-Thr49.

In the description and examples, reference is made to the following sequences:

SEQ ID NO: 1:	sequence comprising the Krix-1 heavy chain CDR regions
SEQ ID NO: 2:	sequence comrpising the Krix-1 light chain CDR regions
SEQ ID NO: 3:	recombinant Krix-1 forward primer heavy chain
SEQ ID NO: 4:	recombinant Krix-1 reverse primer heavy chain
SEQ ID NO: 5:	recombinant Krix-1 forward primer light chain
SEQ ID NO:6:	recombinant Krix-1 reverse primer light chain
SEQ ID NO: 7:	Asn47Gln Krix-1 forward primer
SEQ ID NO: 8:	Asn47Gln Krix-1 reverse primer
SEQ ID NO: 9:	Thr49Ala Krix-1 forward primer
SEQ ID NO: 10:	Thr49Ala Krix-1 reverse primer
SEQ ID NO: 11:	Asn47Glu Krix-1 forward primer
SEQ ID NO: 12:	Asn47Glu Krix-1 reverse primer
SEQ ID NO: 13:	Asn47Asp Krix-1 forward primer
SEQ ID NO: 14:	Asn47Asp Krix-1 reverse primer

DEFINITIONS

The term "Antibody" ("Ab") as used herein refers to intact monoclonal or polyclonal antibody molecules as well as fragments thereof comprising:

a) either both heavy and light chains, (such as Fab, $F(ab)_2$, $F(ab')_2$) or single heavy or light chains (e.g. light chain dimers), optionally including their constant region (or parts thereof), or optionally minor modifications (such as allotypic variants) of that constant region;
b) parts, thereof, in particular the specificity-determining parts thereof, i.e. the variable regions of the antibodies
c) subparts thereof, in particular the hypervariable parts thereof, such as peptides made up of stretches of amino acids comprising at least one CDR, optionally with adjacent framework sequences, e.g. of up to about 10 amino acid sequences at one or both CDR.

Optionally, according to the present invention, antibodies are IgG antibodies, particularly IgG1. F(ab')2 refers the antibody fragment obtainable after pepsin cleavage and is built up of both light chains and parts of the heavy chains disulfide linked via the hinge region. The Fab fragment is obtainable from the intact antibody or from the F(ab')2 by papain digestion of the hinge region and contains a one light chain and one part of the heavy chain. Fragments of antibodies can also be obtained by synthesis or by recombinant methods described in the art.

The term "native antibody" as used herein refers to an inhibitory antibody which is obtained through different methods known in the art and which of which the glycosylation is subsequently modified according to the present invention. The glycosylation of the 'native antibody' is the glycosylation as observed under standard conditions, e.g. for a natural (such as a human) antibody obtained from a cell line (eg immortalized B cells), it refers to the glycosylation of the antibody as observed under standard culturing of said cell line, i.e. unmodified by the presence of enzymes or by mutations. Preferably, such a native antibody is a wild-type antibody, but it is envisaged that a genetically engineered antibody can be further modified and thus in such a context the glycosylation of the native antibody corresponds to the original glycosylation in the antibody as originally constructed and produced. In the context of the present invention, when glycosylation of an antibody derived from Krix-1 is compared to the native

antibody, a comparison to the antibody as obtained from the Krix-1 cell line (deposited as LMBP 5089CB), under standard cultivation conditions is intended.

The term "antibody with modified glycosylation" as used herein refers to antibodies which have been engineered or produced in a way that their glycosylation differs from that of the native antibody, meaning that certain extra carbohydrates are present or certain carbohydrates are missing relative to the native antibody or a combination thereof at different positions. In the context of the present invention the modifications in the glycosylation of the antibodies occur in the variable region (i.e. VH and/or VL) of the antibodies.

An "inhibitory antibody" or an "antibody with inhibitory activity" as used herein refers to an antibody which inhibits the activity of its target protein at least partially. According to a particular embodiment of the present invention, the inhibitory antibodies inhibit the interaction of their target protein with another protein. A specific embodiment of an inhibitory antibody is an anti-Factor VIII antibody, more particularly an antibody inhibiting the binding of FVIII to other factors such as vWF and/or phospholipids. Preferably, the antibodies are directed against the C2 domain of FVIII. Inhibitory antibodies can be either alloantibodies against exogenous FVIII. Inhibitory antibodies can be of human or animal origin. In the context of antibodies inhibiting the activity of factors of the coagulation cascade, also referred to as anti-coagulant antibodies herein, the maximal inhibitory of the antibody may be critical, complete inhibition of coagulation may cause side-effects such as uncontrolled bleeding.

A variable maximal inhibitory activity as used herein relates to a maximal inhibitory activity, as defined for an antibody or a mixture of antibodies according to the present invention, which can be modified. For instance, according to the present invention the maximal inhibitory activity of an antibody against FVIII is decreased, by modification of the glycosylation, more particularly by deglycosylation of the variable region. Thus, antibodies with variable maximal inhibitory activity are obtained. It is understood that in

the context of the present invention for an antibody to be considered as inhibitory, its inhibitory effect should be at least 1%.

Complementarity determining regions (CDR) in the present invention refers to the hypervariable amino acid sequences within antibody variable regions which interact with the epitope on the antigen. In one embodiment of the present invention the CDR regions are the CDR1, CDR2 and CDR3 regions of the variable light (VL) and heavy (VH) chains respectively (L1, L2, L3 and H1, H2, H3 respectively) of antibodies directed against an element of the coagulation cycle.

"Humanized antibody" as used herein, refers to non-human antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody.

A "Reshaped human antibody" or a "Human hybrid antibody" as used herein, refers to a human antibody in which amino acids in the antigen binding regions have been replaced with sequences in accordance with the present invention, e. g. CDR's, or other parts of variable regions which have been derived from the repertoire of human antibodies.

Sequence comparisons. Comparisons of protein or nucleotide sequences are designated in terms of sequence identity or sequence similarity. Where in accordance with the present invention comparisons are made between amino acid sequences of two VH regions or of two VL regions, or comparisons are made between two nucleotide sequences encoding CDRs , or sequences comprising CDRs, the level of sequence identity or similarity between two sequences may include having at least 80%, preferably at least 80% more preferably at least 90%, even more preferably at least 95% and most preferably at least 99% sequence identity or similarity between two sequences.

Nucleotide or amino acid sequences which are "identical" means that when two sequences are aligned, the percent sequence identity, i.e. the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids

in the shorter of the sequences, is higher than 80%, preferably at least 90%, even more preferably at least 95%, most preferably at least 99%, more specifically is 100%. The alignment of two nucleotide sequences is performed by the algorithm as described by Wilbur and Lipmann (1983, Proc. Natl. Acad. Sci. U.S.A. 80:726) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4.

Two amino acids are considered as "similar" if they belong to one of the following groups GASTCP; VILM; YWF; DEQN; KHR. Thus, sequences which are similar means that when the two protein sequences are aligned the number of positions with identical or similar nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the sequences, is higher than 80%, preferably at least 90%, even more preferably at least 95% and most preferably at least 99%, more specifically is 100%.

Nucleotide sequences are considered as "similar" if they encode the similar or identical amino acid sequences, (i.e. between two nucleotide sequences encoding identical proteins the variation is within the bounds of genetic code degeneracy). "modified" denotes any protein (or polypeptide) molecule in which a single or a number of amino-acids have been either substituted by any other amino-acid residue or deleted. Such amino-acid substitution or deletion can be located anywhere in the protein molecule. It also denotes protein molecules in which amino-acid residues have been substituted and/or deleted at more than a single location. In the latter case, any combination of substitution and deletion can be considered. It also refers to polymorphisms (i.e. the regular and simultaneous occurrence in a single interbreeding population of two or more alleles of a gene, where the frequency of the rarer alleles is greater, typically greater than 1%, than can be explained by recurrent mutation alone.)

The term "Carbohydrate cleaving or transforming enzymes" as used herein relates to enzymes that are able to cleave carbohydrates, parts of a carbohydrate structure and/or different molecules coupled thereto (like N-acetyl) from a protein, a peptide or a certain amino acid therein or that are able to covalently bond carbohydrates to amino acids or to other carbohydrates on a protein or peptide. Examples of such enzymes are the peptide N-4(N-acetyl-beta-glucosaminyl)asparagine amidase F (PNGase F), also called N-glycosidase F, and β-galactosidase.

"Antigen binding region" as used herein refers to the region of an antibody involved in the binding of the antigen. More in particular, the antigen binding region can be determined as the amino acids and their substituents which contact through non-covalent bonding amino acids or molecules of the target protein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described with reference to certain embodiments and to certain figures but the present invention is not limited thereto but only by the claims.

It was recently observed that a recombinant antibody produced in CHO cells inhibited FVIII significantly differently from the antibody produced in a human lymphoblastoid cells. This unexpected observation indicated that posttranslational modification could modulate the inhibitory activity of Krix-1. The identification of a glycosylation site in the variable region of Krix-1 further showed that glycosylation of the variable region could modulate the activity of Krix-1.
The activity of Krix-1 treated with enzyme removing carbohydrate structures was therefore investigated. Deglycosylation of Krix-1 dramatically modified its inhibitory activity (down to about 60%). However, the affinity of Krix-1 for FVIII was not changed significantly. These observations were unexpected, because it is well known by those skilled in the art that glycosylation of the variable part of an antibody can increase or reduce the affinity of the antibody for its antigen but it has never been reported before that glycosylation can modulate the function of an antibody otherwise than by altering the affinity or specificity. The ability to modify the activity of an antibody without significantly modifying its specificity or affinity brings the important advantage that mixing different "glycan-modified" antibodies with the same affinity and specificity allows to generate antibody preparation with different maximal inhibitory activity ("plateau"). Moreover, the limitation of the modification of glycosylation to the variable

region ensures that other characteristics of the antibody known to be influenced by glycosylation of the constant region (e.g. half-life) are not affected.

The fact that glycosylation of the antigen binding site of antibodies could alter their inhibitory capacity without significantly modifying their affinity was confirmed by the observation that recombinant antibodies carrying a point mutation in the glycosylation site inhibited only 40% FVIII activity. More interestingly, mixing the different modified forms of recombinant mAb-Krix-1 made it possible to obtain combinations with varying plateaus of FVIII inhibition . Accordingly, this strategy allows the production of an anticoagulant FVIII preparation inhibiting FVIII in a very large therapeutic range, allowing to select the best ratio between anticoagulant action and bleeding risks. The long half-life of the antibodies allows to obtain these target inhibition for prolonged period of time.

The present invention that the glycosylation of KRIX-1 does not significantly change the affinity for the target coagulation factor and thereby does not change the binding to the target coagulation factor demonstrates that the mechanism by which the glycosylation of KRIX-1 affects the inhibitory activity of KRIX-1 is by altering the interaction of the target coagulation factor with other proteins of the coagulation cascade at a site in the variable region. We therefore describe a method based on modification of the glycosylation site of the variable region of an antibody resulting in a modification of the inhibitory effect of the said antibody on the interaction(s) of the ligand(s) recognized by an antibody with other proteins or reagents interacting with the said ligand.

Based on these observations, the present invention provides a variety of antibodies and fragments thereof, which are characterized by modifications of the glycosylation of the antigen binding site of native antibodies resulting in a modification of the maximal inhibitory activity exerted by the antibodies without significantly altering their affinity or specificity for their target protein. In a certain embodiment of the present invention, the antibodies are directed against an element of the coagulation system, more in particular against Factor VIII.

According to the present invention, modification of the glycosylation of an antibody in the variable region does not necessarily significantly affect its affinity for the antigen. More particularly, according to the present invention, the affinity of the antibody is changed due to modifications in glycosylation in such a way that the dissociation konstant (Kd) of the antibody is modified by a factor less than 3, which is considered as asubstantially unaffected affinity of the antibody for the antigen; preferably the K_D of the antibody is modified by a factor less than 2.5, more preferably less than 2, especially preferably less than 1.5. Affinity of an antibody for its antigen can be measured in different ways known to the person skilled in the art. According to a particular embodiment of the present invention affinity of the antibody for the antigen is measured by surface plasmon resonance analysis, as described herein.

The present invention thus relates to antibodies with modified glycosylation, a modified inhibitory activity and asubstantially unaffected affinity for their target protein. The present invention further relates to the use of said antibodies as a medicine. The present invention also relates to methods of preparing such antibodies, a method of selecting such antibodies and pharmaceutical compositions comprising them. The present invention also relates to said antibodies in mixtures with other antibodies, such as with their native antibody.

In a particular embodiment of the present invention, the antibodies are directed against a "protein which is involved in a complex". Proteins involved in a complex can be defined as proteins which interact with one other element next to their target during the performance of their specific activity. Such other elements can be proteins, peptides, phospholipids, salts, lipids, nucleic acids, organic molecules, et. An example of a protein involved in a complex is Factor VIII which interacts with phospholipids and/or the Von Willebrand Factor upon performing its activity (FVIIIa).

In a more particular embodiment of the present invention, the antibodies are directed against an element of the haemostasis system. Elements of the haemostasis system include the factors of the coagulation cascade and include factors such as Factor V,

Factor VII, Factor VIII, Factor X, Factor XI, thrombin, the Von Willebrand Factor and other elements of the coagulation cycle and their active derivatives. In a more particular embodiment of the invention, the antibodies are directed against Factor VIII, more particularly against the C1 or C2 domain of factor VIII, although it is not limited thereto.

The invention further relates to the use of said antibodies in the manufacture of a medicament useful for the treatment of subjects suffering from a certain disorder wherein a protein involved in a complex is involved. Such diseases can be selected from cardiovascular diseases, cancer, autoimmune diseases or immunology related disorders, inflammatory, metabolic, haematological or respiratory diseases. The invention further relates more in particular to the treatment of subjects suffering from a coagulation disorder, more in particular from a venous thromboembolic disease with said antibodies. Venous thromboembolic disease includes deep disorders such as vein thrombosis, pulmonary embolism and atrial fibrillation. The present invention furthermore relates to a method of treatment of coagulation disorders by using said antibodies.

The present invention therefore relates to antibodies with a modified glycosylation and a modified maximal inhibition, but a substantially unaffected affinity for its target protein. The antibodies can be completely deglycosylated or partially. The antibodies can be modified to bear different carbohydrates at different sites or can have an increased glycosylation. In a particular embodiment of the present invention, the maximal inhibitory capacity of the antibodies can be decreased or increased. In a particular embodiment of the invention, the inhibitory capacity of the antibodies of the invention are sub-maximal (≤ 99%) and can range between 20% or 99%. More in particular, the inhibitory activity of said antibodies can be 80%, 70%, 60%, 50%, 40%, 30% or 20%. The inhibitory activity of said antibodies can be measured by any known method in the art. For the field of coagulation, the inhibitory activity of for example antibodies against FactorVIII can be determined by using the Bethesda assay.

The present invention also relates to a method for selecting antibodies with a modified glycosylation, a modified inhibitory capacity and an unaffected affinity, characterised in

that the method comprises the steps of measuring the inhibitory capacity and the affinity of an antibody, modifying the glycosylation of the antibody and again measuring the inhibitory capacity and the affinity of modified antibody. The method of selecting the antibodies of the present invention starts with the preparation and development of an inhibitory native antibody against a certain target protein. This native antibody can be directed to any antigen of the target protein, more in particular to antigens near to the active site of the target protein or to sites of the protein important for the activity of the target protein. More particularly, the antibodies can be directed against the site being at a predetermined distance from a physiologically functional site of the target protein. The next step in the selection procedure is to modify the glycosylation of the native antibody by different methods (enzymatic cleavage, enzymatic adding of carbohydrates, mutations, etc.). The following step would be to measure the inhibitory activity of the modified antibodies.

In a particular embodiment of the present invention, said antibody with a modified glycosylation, a modified inhibitory capacity and unaffected affinity is directed against Factor VIII. In a particular embodiment of the invention, said antibodyis a recombinant antibody prepared in suitable host cells, such as CHO cells. Alternatively, said antibody is prepared by site-directed mutagenesis, more in particular said antibody has no N-glycosylation site in the variable region. In another alternative, said antibody is prepared by exposing antibodies to carbohydrate cleaving enzymes. In a yet more particular embodiment, said antibody has a factor VIII inhibitory capacity between 20% and 90%, more in particular between 30% and 80%, yet more in particular between 40% and 70% and still more in particular between 50% and 60% or any combination thereof.

In another embodiment of the present invention, said antibody of the present invention and directed against Factor VIII is derived from a cell line called Krix-I or a cell line producing antibodies with the same characteristics. In a more particular embodiment said antibody is derived from Krix-1 or a fragment thereof or a recombinant produced analogue of a modified Krix-1, more in particular said antibody has an amino acid similarity of at least 80%, preferably at least 90% with Krix-1 or a fragment thereof. In a yet more particular embodiment of the present invention, said antibody is the

recombinant antibody Krix-1 or a fragment thereof, produced in CHO cells and with an inhibitory activity of around 84%. In a yet more particular embodiment, said antibody is a mutant of Krix-1 with mutated positions Asn47 to Thr49, more in particular with Asn47 changed in Gln47 (LE2E9Q), Glu47 (LE2E9E) or Asp47 (LE2E9D) and/or Thr49 in Ala49 (LE2E9A). The present invention also relates to antibodies derived from Krix-1 by incubating the native antibody with carbohydrate cleaving enzymes such as N-glucosidase-F, more particularly to antibodies obtained in this way and with an inhibitory activity of around 50%. The antibodies derived from Krix-1 can have an inhibitory activity of at least 20%, more particularly at least 40%, 50% or 80%.

These antibodies and fragments thereof carrying modified glycosylation in the variable region, optionally in or in the proximity of a CDR have the advantageous properties that they exhibit therapeutically useful maximal inhibitory activity different from the native antibody, while inactivating the target protein only partially even when the antibody is in a molar excess, like the native antibody. These glycan-modified antibodies and fragments thereof are therefore useful as agents for obtaining only a partial inhibition a target protein, more in particular as anticoagulant agents allowing to achieve desirable partial inhibition of a coagulation factor out of reach of the native antibody in the case of an antibody directed against an element in the coagulation system.

The fact that the affinity of the modified antibodies of the invention is not significantly changed is of critical value for their use in mixtures and means that the modified antibodies will, similarly to the native antibody displace the natural ligand. This allows the formulation of mixtures of antibodies in order to obtain a well-defined inhibitory activity. More particularly, this is of interest in the field of anti-coagulant antibodies, where the maximal inhibitory activity may be critical. For instance, in some clinical settings, anti-FVIII antibodies with different inhibitory activities could be required. For example, short term prophylaxis of thrombosis following surgical intervention may be optimally treated with drugs with a potency different from those required for the treatment of a chronic condition such as atrial fibrillation.

In a certain embodiment of the present invention, the modified antibody is used in a mixture with other antibodies directed against the same target protein, yet more in particular directed against the same antigen or derived from the same cell line. This mixture can comprise the native antibodies together with modified antibodies directed against the same target protein or the mixture can contain two antibodies modified in a different way in their glycosylation pattern. The different parts of the mixture can be mixed in such quantities so that any wanted inhibitory activity can be obtained.

The present invention relates to a method for preparing antibodies with a modified glycosylation and modified maximal inhibition, but without an altered affinity or specificity for their target protein. Therefore, the present invention relates to a method of producing said antibodies comprising the step of exposing antibodies to carbohydrate cleaving or transforming enzymes. Alternatively, the method of preparation of the present invention comprises the step of producing the antibodies in cell lines with suitable glycosylation enzymes or by modifying the cell culture conditions to modify the activity of the glycosylation enzymes of the cell line producing the antibodies. In another ambodiment of the present invention, the method of preparing said antibodies comprises the step of genetically modifying the antigen binding site of the antibody in order to remove or introduce glycosylation sites, for example by site-directed mutagenesis.
In a particular embodiment of the invention, the glycosylation of the antibody is modified in its antigen binding region or in the amino acids in the proximity of the antigen binding region.

The present invention thus relates to antibodies which have a modified glycosylation pattern relative to the native antibody.
The native antibody can be prepared according to known methods in the art.
Monoclonal antibodies against a certain target protein can be produced by any technique which provides the production of antibody molecules by continuous cell lines in cultures such as the hybridoma technique originally developed by Kohler and Milstein (Kohler and Milstein Nature 1975. 256: 495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 1983, 4: 72), the EBV-

hybridoma technique to produce human monoclonal antibodies (Cole et al. 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. Pp 77-96) and the like, all are within the scope of the present invention.

The monoclonal antibodies may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies or even from any other kind known in the art, such as coming from cammels or lamas. Human monoclonal antibodies may be made of any numerous techniques known in the art (e.g. Teng et al, Proc. Natl. Acad. Sci. U.S.A. 1983, 80: 7308 - 7312; Kozbor et al., Immunology Today 1983, 4: 72-79, Olsson et al, Meth. Enzymol. 1982, 92: 3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al, Proc. Natl. Acad. Sci. U.S.A. 1994, 81: 6851, Takeda et al. Nature 1985, 314: 452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of the target proteins. For the production of antibody, various host animals can be immunized by injection with a specific protein, or a fragment or derivative thereof, including but not limited to rabbits, mice and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyosl, polyanions, peptides, oil emulsions, keyhole limpet hemocyanisn, dinitrophenols, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a selected protein epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g. Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory , New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments, which contain the idiotype of the molecule, can be generated by known techniques. For example, such fragments include but are not limited to the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule, the Fab' fragments which can be generated by reducing the disulfide

bridges of the F(ab')2 fragment and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Antibody molecules can be purified by known techniques, e.g. immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof.

The antibodies of the present invention can be prepared by conventional hybridoma techniques, phage display, combinatorial libraries, immunoglobulin chain shuffling, site directed mutagenesis and humanization techniques to generate novel antibodies with limited maximal inhibitory activity.

The present invention further provides modified antibodies derived from native monoclonal antibodies being produced by on purpose immunization in animals, preferably in mouse, for instance by injecting human Factor VIII in mice and then fusing the spleen lymphocytes with a mouse myeloma cell line, followed by identifying and cloning the cell cultures producing anti-factor VIII antibodies as further as described in WO97/26010 and/or WO 01/04269. More particularly, in the context of the present invention, epitopes of the antigen flanking the active site of said antigen can be used for immunization, in order to promote the development of antibodies in which the inhibitory effect of the antibody is not linked directly to the binding of the antigen through the CDR.

The present invention provides for antibodies which are modified in their glycosylation pattern. The modification of the glycosylation of native antibodies can be obtained through different methods known in the art. Modification of the glycosylation pattern in the antigen binding site of the antibodies of the present invention can be achieved by enzymatic treatment of purified antibodies. Alternatively, modification of the glycans of the antibodies of the present invention can be achieved by producing the antibodies in cell lines with suitable glycosylation enzymes or by modifying the cell culture conditions to modify the activity of the glycosylation enzymes of the cell line producing the antibodies. Alternatively, the antibodies of the present invention can also be produced by

genetically modifying the antigen binding site of the antibody in order to remove or to introduce glycosylation sites.

Many carbohydrate cleaving or transferring enzymes can be applied in order to modify the glycosylation pattern of a native antibody. The glycosylation can be increased or decreased completely or partially. In a particular embodiment, the modificatin is obtained in the antigen binding region of the antibody. Enzymes can be applied on a native antibody in a different order and under variable circumstances (concentrations, time, temperature, buffer, etc.) in order to obtain antibodies with different glycosylation patterns.

Enzymes such as peptide N-4(N-acetyl-beta-glucosaminyl)asparagine amidase F (PNGase F), also called N-glycosidase F can be used. This enzyme has a broad specificity, and it releases nearly all known N-linked oligosaccharide chain from proteins (Plummer 1984). This enzyme releases tetra- and penta-antennary chains. It is noteworthy that the activity of the enzyme can only be predicted when the glycoprotein is fully denatured. Accordingly, the activity of the enzyme on an intact antibody must be controlled in each case. Methods to control the deglycosylation of the antibody are described in Current Protocols in Protein Science, Ed. G. Taylor, Unit 12.4; John Wiley & Sons, Inc.

In particular, the glycosylated and deglycosylated antibodies are compared by isoelectrofocusing.

Truncated glycoforms of IgG can be generated by sequential enzymatic treatment as described in Mimura Y et al. (J Biol Chem. 2001;276:45539-47) and summarized in Figure 1.

Sialic acids are the terminal sugars on many N- and O-linked oligosaccharides. To remove sialic acid, the native IgG in acetate buffer, pH 5.0, are exposed to sialidase (such as the sialidase from *Arthrobacter ureafaciens*, Roche Molecular Diagnostics, East Sussex, UK) at 37°C for 24h. Removal of sialic acids results in an increase in the isoelectric point of the protein. IEF can therefore be used to control removal of sialic acids.

Upon removal of sialic acids, galactose can be removed by treatement with β-galactosidase (Diplococcus pneumoniae, Roche Molecular Biologicals) in acetate buffer, at 37°C for 24h. Following removal of sialic acid and galactose, N-acelylglucosamine can be cleaved by treatment with N-acetyl-β-D-glucosaminidase (D pneumoniae, Roche, Molecular Biochemicals) in 37°C for 24h. Mannose residues can then be removed by treatment with α-mannosidase (jack bean, Glyko, Oxfordshire, UK) at 37°C for 48h (Mimura Y et al., J Biol Chem. 2001;276:45539-47.

Different types of sialidase have also been described. The sialidase (neuraminidase) from *Arthrobacter ureafaciens* releases both α2,3- and α2,6- linked sialic acids, whereas the sialidase from the Newcastle disease virus releases only α2,3 linked sialic acids (Jassal et al., 2001). The endoglycosidase F2 cleaves the bound between the two GlcNAc residues in the core region, leaving one GlcNAc still bound to the protein. Endoglycosidase F2: preferentially releases biantennary complex-type oligosaccharides chains from glycoproteins but does not cleave tri- or tetraantennary chains

Endoglycosidase F3: another endoglycosidase with a narrow substrate range: it cleaves triantennary chains. A core fucosylated biantennary chain is the only other demonstrated substrate. It does not cleave *high-mannose hybrid, nonfucosylated biantennary or tetraantennary chains*. All linkages which can be cleaved by endoglycosydase F2 and F3 are not exposed in a mature antibody. Methods suitable to determine whether an antibody can be usefully modified by these endoglycosidase include SDS-PAGE, lectin binding methods using Ricinus communis agglutinin-1 and IEF as described above.

Conversely, glycan residues can be enzymatically added to carbohydrate expressed in the variable part of the antibody. For example, treatment with sialidase as described above can be followed by treatment with galactosyl-transferase and UDP-Gal in a suitable buffer (Krapp et al.,2003). The modified antibody are then homogenous for galactosylation of the carbohydrate chain (biantennary digalactosylated glycoform) (Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. J Mol Biol. 2003;325:979-89).

The purification of antibodies carrying different oligosaccharides is also known to persons skilled in the art. The antibodies carrying different oligosaccharides can be purified by lectin affinity chromatography, such as Concanavalin A (binding to a bisecting GlcNAc). Aleuria aurantia differentiates on the basis of core fucosylation. *Ricinus communis agglutinin 1* fractionates according to the number of galactose residues because this lectin exhibits specific affinity to oligosaccharides ending with galactose (Youings et al. Biochem J, 1996, 314: 621).

All carbohydrate residues are not exposed in a mature antibody. Methods suitable to determine whether an antibody can be usefully positively or negatively purified using the above lectin are well known by those skilled in the art. Unbound antibody can be tested to determine their inhibitory activity on FVIII using the Bethesda method (Kasper 1976). Similarly, the activity of the antibody captured on the column and eluted using a suitable buffer, can be tested using the Bethesda method (Kasper 1976).

An alternative method for modifying the glycosylation of an antibody is to generate recombinant antibodies with modified glycosylation pattern by producing recombinant antibodies in cell lines selected as a function of their repertoire of glycosylation enzymes. Chinese Hamster Ovary cells (CHO) are well known example of such a cell line.

Although CHO cells have most of the human repertoire of glycosylation enzymes, they are deficient in particular glycosyltransferases. In particular, the α2,6-sialyl-transferase gene (1,2) is not expressed endogenously in CHO cells. This enzyme adds terminal galactose sugars with sialic acid in the α2,6 position on the Galβ1, 4GlcNAc-R sequence. However, CHO cells express a functional α2,3-sialyl-transferase so that the terminal sialic acids are in α2,3 linkage to galactose. Alpha-3/4 fucosyltransferase is also not synthesized by these cells (Grabenhorst E et al., Glycoconj J. 1999;16:81).

Another method to produce recombinant antibody with modified glycosylation pattern is to use a cell line genetically modified to express glycosylation enzyme from other strains. In particular, a CHO-K1 cell line transfected with an α2,6-sialyltransferase gene cloned from another strain can be used (Jassal et al., Biochem Biophys Res Comm, 286: 243, 2001). In a particular embodiment of the invention, the Factor VIII inhibitory activity of

the recombinant antibody produced in such a cell line can then be evaluated in the Bethesda assay using the modification of the Nijmegen method as described above.
Furthermore, also culture conditions can be exploited to modify the glycosylation of the recombinant antibody. The concentration of dissolved oxygen at steady state in serum free culture has an effect on glycosylation of antibody. The extent of galactosylation is reduced with reduced dissolved oxygen concentrations (Kunkel JP, Jan DC, Jamieson JC, Butler M. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J Biotechnol. 1998;62:55-71). Supplementing the medium with more than 20 mM N-acetylglycosamine can also induce new antibody glycoforms (Tachibana H, et al. Generation of specificity-variant antibodies by alteration of carbohydrate in light chain of human monoclonal antibodies. Biochem Biophys Res Commun. 1992;189:625-32; Tachibana H, et al. Modified antigen-binding of human antibodies with glycosylation variations of the light chains produced in sugar-limited human hybridoma cultures. In Vitro Cell Dev Biol Anim. 1996;32:178-83. Glucocorticoid hormones and interleukin 6 are involved in the modulation of protein glycosylation (Canella A and Margni RA, Hybrid Hybridomics 2002, 21:203).
Therefore, selection of the cell line and cell culture conditions can have a big influence on the glycosylation pattern.

Another alternative to the enzymatical modifications and the recombinant production of the antibodies is to use (site-directed) mutagenesis. New glycosylation sites can be introduced or existing glycosylation sites can be removed with this technique. N-glycosylation sites can be introduced by site directed mutagenesis in the antigen binding site of the antibody. Amino-acid stretches containing N-glycosylation sites can be selected in the published sequences of antibodies glycosylated in the antigen binding site. The selection of antibodies inhibiting FVIII activity in a desirable manner can be made using the Bethesda assay (Kasper 1976). The protein structure can also be modified to indirectly modify glycosylation (Lund J et al., J Immunol. 1996;157:4963; Lund J et al., J. Eur J Biochem. 2000;267:7246). Site-directed mutagenesis is a well known method for the person skilled in the art.

The present invention also provides fragments of any of the above mentioned monoclonal antibodies such as Fab, Fab', F(ab')2, scFv, CDR's, single variable domains as well as derivatives, homologs and combination of these.

In a particular embodiment of the present invention, the antibodies are directed against elements of the coagulation system, more in particular against Factor VIII.
The present invention therefore relates to antibodies derived from Krix-I, more in particular to antibodies modified in their glycosylation pattern derived from Krix-I and with a modified factor VIII inhibitory activity. Particularly, the glycan-modified antibodies are derived from the human monoclonal antibody Krix-1, fragments thereof or contains one or several complementary determining region thereof. Exemplary antibodies with modified glycosylation site are antibodies produced by treatment of Krix-1 with N-glycosidase F. Particularly are genetically modified antibodies containing the mutations Asn47Glu and Thr49Ala in the CDR1 of the heavy chain of Krix-1.
Modification of the glycosylation modifies the Factor VIII inhibitory activity of mAb-Krix-1. A particular method to evaluate the inhibitory activity of an antibody with modified glycosylation pattern is the Bethesda assay (ref Kasper 1976) using the modification of the Nijmegen method (Verbruggen et al.). In this assay, normal pooled plasma, used as a FVIII source, is mixed with an equal volume of antibody. After an incubation of 2h with antibody, the residual FVIII activity was measured by a Factor chromogenic or clotting assay.

The cell line named Krix-1 was disclosed in WO 01/04269 and was deposited with the BCCM/LMBP (Belgian Co-ordinated Collections of Microorganisms/Plasmid Collection Laboratorium voor Moleculaire Biologie, University of Gent K.L. Ledeganckstraat 35, B-9000 Gent, BE under accession number LMBP 5089CB on July 1, 1999.
By partial inhibition of FVIII activity, we mean that the maximal inhibition of Factor VIII activity by a certain antibody is lower or equal to 99%, as determined using a suitable test method, such as the Bethesda assay. The residual Factor VIII activity is then measured using a coagulation or a Chromogenic Factor VIII assay. A suitable

chromogenic assay is for example the Coatest[R] (Chromogenix-Instrumentation Laboratory SpA, Milano, Italy).

The antibodies described in this invention can be useful in the therapy or prevention of different diseases. In the case of antibodies directed against elements of the coagulation system, the antibodies are useful in therapeutic and pharmaceutical compositions for thrombotic disorders associated with myocardial infaction, unstable angina, atrial fibrillation, stroke, renal damage, pulmonary embolism, deep vein thrombosis, percutaneous translumenal coronary angioplasty, disseminated intravascular coagulation, sepsis, transplants, shunts.

The present invention further provides a pharmaceutical composition for the prevention or treatment of coagulation disorders in animals, more particularly in humans, comprising, as an active ingredient, the antibody and fragments and modified versions thereof with a modified glycosylation pattern and inhibitory activity but unaffected affinity, such as disclosed hereinabove, in admixture with a pharmaceutically acceptable carrier.

Suitable pharmaceutical carriers for use in the pharmaceutical compositions of the invention are described for instance in Remington's Pharmaceutical Sciences 16 ed. (1980) and their formulation is well known to those skilled in the art. They include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like. Additional ingredients may be included in order to control the duration of action of the monoclonal antibody active ingredient in the composition.

Control release compositions may be obtained by selecting appropriate polymer carriers such as for example polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinyl acetate copolymers, methylcellulose, carboxymethylcellulose, protamine sulfate and the like. The rate of drug release and duration of action may also be controlled by incorporating the monoclonal antibody active ingredient into particles,e. g.

microcapsules, of a polymeric substance such as hydrogels, polylactic acid,hydroxymethylcellulose, polymethyl methacrylate and the other above-described polymers. Such methods include colloid drug delivery systems like liposomes, microspheres, microemulsions, nanoparticles, nanocapsules and so on. Depending on the route of administration, the pharmaceutical composition comprising the active ingredient may require protective coatings. The pharmaceutical form suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and mixtures thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The above detailed description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1: Schematic representation of the biantennary structure most commonly found in the antigen binding part of antibodies. NeuAc= N-acetylneuramic acid (sialic acid); Gal = galactose; GlcNac = N-acetylglucosamine; Man = mannose; Fuc = fucose; Asn = asparagine.

Figure 2: Schematic representation of the removal of glycan by sequential enzymatic treatment.

To remove sialic acid, the native IgG are exposed to sialidase. Upon removal of sialic acids, galactose can be removed by treatement with β-galactosidase. Following removal of sialic acid and galactose, N-acelylglucosamine can be cleaved by treatment with N-acetyl-β-D-glucosaminidase. Mannose residues can then be removed by treatment with α-mannosidase.

Figure 3: Graph of experimental results showing the inhibitory activity of native and deglycosylated KRIX-1, in accordance with an embodiment of the invention.
KRIX-1 was deglycosylated by treatment with N-glycosidase-F. To assess the inhibitory activity of native (NAT; closed symbol) and deglycosylated KRIX-1 (DEG; open symbol), one volume of antibody at various dilutions was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The residual FVIII activity was then measured in a chromogenic assay.

Figure 4: Graph of experimental results showing that mixing deglycosylated KRIX-1 with native KRIX-1 reduces the maximal "plateau" inhibition of FVIII, in accordance with an embodiment of the invention.
Normal plasma was incubated for 2h at 37°C with various concentrations of Krix-1, deglycosylated Krix-1, and mixtures of native and deglycosylated Krix-1 at a ratio of 4.5 and 1.5 native versus deglycosylated antibody. After a 2h incubation period at 37°C, the residual FVIII activity was measured in a FVIII chromogenic assay.

Figure 5: Graph of experimental results showing the inhibitory activity of CHO-recKRIX-1 and KRIX-1 on FVIII activity in plasma, in accordance with an embodiment of the invention.
To assess the inhibitory activity of the antibody produced by the human cell line(KRIX-1) and the recombinant antibody produced in CHO (CHO-recKRIX-1), one volume of antibody at various dilutions was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The residual FVIII activity was then measured in a chromogenic assay.

Figure 6: Graph of experimental results showing the effect of KRIX-1 and CHO-recKRIX-1 on vena cava thrombosis in mice, in accordance with an embodiment of the invention.
Thrombus was induced in the inferior vena cava 16 hours after subcutaneous administration of 150 μg KRIX-1 and CHO-recKRIX-1 or saline. Animals were sacrificed after 4 hours. Five transverse segments at 0.5 mm intervals through the

infrarenal vena cava were scored 1 if thrombus was present or zero if absent, and the scores were summed.

Figure 7: Graph of experimental results showing that KRIX-1, CHO-rec-KRIX-1 protect against penile thrombosis and priapism in mated $AT^{m/m}$ males, in accordance with an embodiment of the invention.

Males were injected twice subcutaneously with vehicle (PBS), or with 100 µg antibody mAb Krix-1 or rec-mAB Krix-1, three days before and on the day of mating. Thrombotic outcome was scored zero if the mice were free of thrombosis at the end of the 8-day follow-up, 1 if microscopic thrombosis without priapism was observed, 2 if macroscopic thrombosis without priapism occurred, and 3 if the males developed severe thrombosis with irreversible priapism. (#) One mouse each in the mAb Krix-1 or rec-mAb Krix-1 treated group was free of macroscopic thrombosis at the end of the experiment but could not be analyzed by microscopy and were therefore scored 1.

Figure 8: Graph of experimental results showing the inhibitory activity of CHO-recKRIX-1 and mutated antibodies with N-glycosylation site in the variable region, in accordance with an embodiment of the invention.

To assess the inhibitory activity of the antibodies, one volume of antibody at various dilutions was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The residual FVIII activity was then measured in a chromogenic assay.

Figure 9: Graph of experimental results showing the inhibitory activity of CHO-recKRIX-1 and Asn47Gln-CHO-recKRIX-1, in accordance with an embodiment of the invention.

To assess the inhibitory activity of the antibodies, one volume of antibody at various dilutions was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The residual FVIII activity was then measured in a chromogenic assay.

Figure 10: Drawing representing the experimental protocol for extracorporeal thrombosis in baboons. Arterial and venous thrombogenic devices. Arteriovenous shunts were

implanted in male baboon femoral vessels. Thrombogenic devices prefilled with saline were incorporated as extension segments into the permanent arteriovenous shunt. Platelet-dependent arterial thrombus was induced by inserting Dacron into the wall of Silastic tubing. Coagulation-dependent venous thrombosis was generated in an expansion chamber. The deposition of autologous radiolabeled platelets was followed with a gamma scintillation camera.

Figure 11: A graph of experimental results showing the inhibition of platelet deposition in the arterial and venous thrombosis chambers before and after administration of Asn47Glu CHO-recKRIX-1, in accordance with an embodiment of the invention.
Platelet deposition was recorded as a function of time in the expansion ("venous") thrombosis chamber (A) and in the Dacron ("arterial") thrombosis chamber (B) incorporated in an extracorporeal arteriovenous shunt implanted between femoral vessels. In the control studies, the devices were kept in place for 60 min or until occlusion of the catheter. The baboons were then treated with a single intravenous bolus of antibody. New thrombogenic devices were placed then for 60 minutes, 1h, 24h after the bolus injection. The extracorporeal shunts were then removed.

EXAMPLES

The present invention will now be described with reference to the following specific, non-limiting examples.

Example 1. Effect of deglycosylation on FVIII inhibition by Krix-1
KRIX-1 (0.5mg/ml in PBS) was mixed with N-glycosidase-F (roche diagnostics Gmbh, Mannheim, Germany) at final concentration of 2U/ml. The mixture was incubated at 37°C during 72 hours under gentle stirring.
The inhibitory activity of native and deglycosylated KRIX-1 was assessed in a Bethesda assay (Kasper et al., 1976). Therefore, one volume of antibody at various dilutions in TBS (Tris 20 mM, NaCl 0,15 M, pH 7,4) was mixed with one volume of a pool of normal human plasma and incubated for 2h at 37°C. The pool of normal plasma had been

constituted by mixing plasma from 10 normal individual and buffered by addition of Hepes (100 mM) to a final concentration of 10 mM. The residual FVIII activity was then measured using a modification of the DADE FVIII chromogenic assay (Dade AG, Marburg, Germany). In this assay, thrombin-activated FVIII accelerates the conversion of factor X into factor Xa in the presence of factor IXa, PL and calcium ions; factor Xa activity is then assessed by hydrolysis of a p-nitroanilide substrate. Reagents, which were reconstituted according to the manufacturer's instruction, comprised bovine factor X (1 mM), factor IXa (0.3 mM) and thrombin (0.3 mM); $CaCl_2$ (30 mM), PL (60 mM), a chromogenic factor Xa substrate (CH3OCO-D-CHG-gly-Arg-pNA.AcOH; 3.4 mM), and a thrombin inhibitor (L-amidinophenylalanine piperidine). Aliquots of 30 µl of plasma/antibody mixture were retrieved at the end of the 2h incubation period and displayed in microtitration plates; 30 µl of the factor X and factor IXa/thrombin reagents were added sequentially. After 90 sec, 60 µl of the chromogenic substrate were added and the incubation extended for 10 min at 37°C. The reaction was then blocked by addition of 30 µl citric acid (1 M), and OD was measured at 405 nm. The residual FVIII activity was determined by comparing the OD_{405nm} of test samples with that obtained with FVIII solutions of known concentrations. The residual FVIII activity was expressed as the percentage of activity measured in plasma aliquots handled and diluted exactly as test samples throughout the entire experiment.

As previously disclosed (WO 01/04269), native KRIX-1 inhibited up to 90% of FVIII activity. By contrast, a maximal inhibition (plateau inhibition) of only 50% was achieved with deglycosylated KRIX-1 (Figure 3).

Example 2. Mixing native and deglycosylated KRIX-1 allows the selection of antibody mixtures inhibiting FVIII to different levels

Mixtures containing different ratio of deglycosylated with N-glycosidase-F versus native KRIX-1 were prepared. Each mixture was diluted to various antibody concentrations ranging between 0,05 and 25 µg/ml. One volume of each dilution was mixed with one volume of a pool of normal human plasma. After 2 hours period incubation at 37°C, the residual FVIII was assesed using a chromogenic assay (Factor VIII Chromogenic assay;

Dade Behring, Marburg, Germany). The native and deglycosylated KRIX-1 inhibited FVIII activity by about 90% and 50%, respectively (Figure 4). By contrast, a mixture of 4,5 native antibody for 1 deglycosylated antibody resulted in a maximal FVIII inhibition of about 80% whereas a mixture containing 1,5 native KRIX-1 for 1 native antibody inhibited about 65% FVIII activity (Figure 4). Mixtures inhibiting FVIII activity to any level comprised between 50 and 90% can be similarly obtained by varying the ratio of native and deglycosylated KRIX-1.

Example 3. Recombinant Krix-1 produced in CHO cells (CHO-recKRIX-1) has a lower FVIII inhibitory activity than Krix-1 (produced by a human lymphoblastoid cell line)

RNA from KRIX-1 EBV-immortalised human B cells was isolated using TRIzol Reagent according to the manufacturer's instructions (Life Technologies). cDNA was synthesised with the SuperScript pre-amplification system for first-strand cDNA synthesis.

The sequences encoding the heavy or light chain were amplified by RT.PCR on mRNA prepared from KRIX-1 cells using the QuickPrep®Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Rosendaal, The Netherlands). Specific PCR primers for the heavy chain were: forward primer 5'-cgggtacc***ccaccATGG***ACTGGACCTGGAGGATC-3' (SEQ ID NO:3) corresponding to nucleotides (nt) 1 to 21 (in capitals) of the cDNA sequence (WO 01/04269 A1), and containing a *Kpn*I site (underlined) for cloning purposes and a Kozak sequence (bold italic); reverse primer: 5'-tatggccgacgtcgac***tca***TTTACCCGGAGACAGGGAGAG-3' (SEQ ID NO: 4)corresponding to nt 1800-1780 (capitals) of the 3' end of the human gamma-4 constant region (accession number K01316) and containing a stop codon (bold italic) and a *Sal*I site (underlined) for cloning purposes. Specific primers for the light chain were: forward primer 5'-cccaagctt***ccaccATGG***AAACCCCAGCKCAGCT-3' (SEQ ID NO: 5) corresponding to nt 1-20 (capitals) of the cDNA sequence (WO 01/04269 A1), and containing a *Hin*dIII site (underlined) for cloning purposes and a Kozak sequence (bold italic); reverse primer: 5'-aaacagcctctaga***cta***ACACTCTCCCCTGTTGAAG-3' (SEQ ID NO: 6)corresponding nt 653-635 of the 3' end of the human kappa constant region (accession number V00557) and containing a stop codon (bold italic) and a *Xba*I site (underlined) for cloning purposes. After sequence verification, the heavy and light

chain sequences were cloned consecutively into the pBudCE4 plasmid (Invitrogen, Merelbeke, Belgium) designed for double gene expression in eukaryotic cells under the control of the EF1-α and the CMV promoter, respectively, using the above indicated restriction sites. The final vector was used for stable transfection of CKO-K1 cells using the FuGENE6 system (Roche Diagnostics, Brussels, Belgium) according to the manufacturer's instructions. The transfected cells were cultured in DMEM (Life Technologies, Paisley, UK) supplemented with 10% FCS, 4 mmol/L glutamine and 80 mg/L gentamicine (Geomycin®, Schering-Plough, Heist-op-den-Berg, Belgium) in the presence of zeocin (0.7 mg/mL selection concentration or 0.35 mg/mL maintenance concentration; Life Technologies, Invitrogen), and were verified for antibody production by ELISA (see below). The cells were adapted to growth in serum-free medium by step-wise reduction of the FCS to 0%, and after clonal dilution, the best producer in terms of functionality (ELISA on huFVIII), as well as expression (ELISA with anti-humanIgG4 detection antibody), was used for batch production.

For detection of anti-FVIII antibodies, rFVIII was insolubilised by incubating plates for 2h at 4°C directly with 50 µl of rFVIII (1 µg/ml) diluted in glycin-buffered saline (GBS). The plates were washed as above and 50 µl of culture supernatant were added for a further incubation of 2h at 4°C. After washing, 50 µl peroxidase-labelled anti-human Fcγ goat IgG (Sigma) diluted 1000-fold in Tris-casein were added. After 2h at RT, the plates were washed again and supplemented with 100 µl OPD. The resulting OD was read at 492 nm in a Emax Microplate Reader (Molecular Devices, Menlo Park, CA). Negative and positive controls were culture medium and IgG purified from a high-titer inhibitor hemophilia A patient, respectively.

The recombinant antibody was purified from the cell culture supernatant by adsorption on immobilized protein A (High-TRAP Protein A, Pharmacia, Uppsala, Sweden). Culture supernatant was passed through a high-TRAP^R protein A (Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min. Bound IgG was eluted with citric acid 100 mM, pH3. After pH neutralisation with Tris pH9, IgG was dialysed against Phosphate buffered saline (PBS). The concentration of proteins was determined with the Bio-Rad assay (Biorad).

The recombinant antibody produced in CHO cells was called CHO-recKRIX-1. Interestingly, the maximal inhibition observed in large excess of this antibody reaches only 75-85% FVIII activity, which is lower than the 85-95% maximal (plateau) inhibition observed when FVIII is incubated with KRIX-1 (produced by the human lymphoblastoid cell line (Figure 5).

Example 4. Prevention of vena cava thrombosis using CHO-recKRIX-1 in mice

Thrombus was produced in the inferior vena cava of adult male wild-type mice (weight 18g – 31g, age 8-10 weeks) using a previously described model (Singh et al., 2002). Mice were anaesthetised with isoflurane, the inferior vena cava was exposed below the renal veins via a median laparotomy and a neurosurgical vascular clip (Braun Medical) was applied for 15 seconds on two occasions, 30 seconds apart to a segment of the vena cava. A 5/0 prolene thread was then placed alongside the vena cava and a stenosis produced by tying a 4/0 silk suture around the vena cava and the prolene thread. The thread was removed to allow blood flow to resume. The abdomen was closed and the animal allowed to recover. After 4 hours, the mice were reanaesthetised and a 1 cm portion of the inferior vena cava (between the point of ligature and iliac bifurcation) was excised and examined for the presence of thrombus. The excised segments were then washed in 10% PBS and soaked overnight in 1% paraformaldehyde. Vessel segments were embedded in paraffin wax and 7 x 10 μm transverse sections were cut at 0.5 mm intervals from the ligature down.

Sections were stained by haematoxylin and eosin, Martius Scarlet Blue (MSB) and a rabbit anti-platelet antibody (Accurate Chemical & Scientific Corporation, Westbury, NY 11590). MSB stains fresh fibrin red or mature fibrin blue/gray, red cells yellow and collagen bright blue. Thrombus size was measured by scoring the 7 sections for the presence of thrombus, giving a score of 1 for the presence and 0 for the absence of thrombus in each. Scores were then added up for each animal. The investigators performing the operations and the microscopic analyses were blinded towards treatment groups.

Thrombosis was induced in three groups of wild-type mice 16 hours after subcutaneous injection of 150 μg of antibody or saline. The statistical significance of differences between groups was evaluated on the presence or absence of thrombus using Fisher's exact test (2-sided). The effects on thrombus size were tested by comparing thrombus scores using the Mann-Whitney U test.

Ten out of 14 mice injected with saline developed a thrombus, visible macroscopically, compared with 0 out of the 7 animals in each of the groups pretreated with either KRIX-1 or CHO-recKRIX-1 ($P < .01$).

Histological analysis identified thrombi in 11 out of 14 control animals and 1, 1, and 2 thrombi, respectively, in animals treated with KRIX-1 or CHO-recKRIX-1 (Figure 6). Accordingly, although CHO-recKRIX-1 inhibits FVIII activity significantly less than KRIX-1, CHO-recKRIX-1 inhibits very efficiently thrombosis and therefore offers a better safety/efficacy profile than the native KRIX-1 antibody.

Example 5. Antithrombotic activity of CHO-recKRIX-1 in mice with type II heparin binding site (HBS) antithrombin deficiency ($AT^{m/m}$)

The antithrombotic efficacy of CHO-recKRIX-1 was evaluated using the thrombotic priapism model in mice with type II heparin binding site (HBS) antithrombin deficiency (Dewerchin et al., submitted).

The mice were previously generated by targeted knock-in of an R48C mutation (corresponding to the "Toyama" R47C mutation in man, abolishing heparin/heparan sulphate binding and cofactor activity (14, 15)) in the HBS of antithrombin (AT) ($AT^{m/m}$ mice), resulting in life-threatening, spontaneous thrombosis at different sites, most prominently in the heart, liver, and in ocular, placental and penile vessels (Dewerchin et al, submitted for publication). The observation of priapism occurrence upon mating of males $AT^{m/m}$ provided the basis to the development of a physiological model of venous thrombosis, providing a defined endpoint and an easy grading of the thrombotic outcome.

Age-matched groups of sexually mature males (2 to 4 months) were subcutaneously injected twice (three days before mating and on the day of mating) with 100 μl of saline

or with 100 μl of a 1mg/ml solution of Krix-1, Asn47Glu or native CHO-recKRIX-1. After the second injection, each male was mated to two wild type Swiss females, which were replaced by two new females on day 3 after mating. The formation of a vaginal mucus plug indicating recent mating was recorded daily for all females, and only the results obtained with males with confirmed sexual activity were incorporated in the analysis. Males were examined daily for development of priapism and were sacrificed when priapism was observed, or at day 8 after initial mating when the experiment was ended. At sacrifice, blood samples were collected for determination of residual FVIII activity and human IgG levels as described above. The penises were dissected and the presence of thrombus IN the dorsal penile vein and corpora cavernosae determined by visual inspection.

After sacrifice, the dissected penises were paraformaldehyde fixed, parafin-embedded and processed for histological analysis. Seven-μm transverse sections were stained with haematoxylin/eosin for microscopic analysis.

Scoring: Thrombotic outcome was scored using four categories: 0, no thrombosis; 1, thrombosis of the penile vein by microscopy; 2, macroscopically visible thrombosis of the penile vein; 3, irreversible thrombotic priapism. When no macroscopically visible thrombus was observed and no histology of the penile vein could be obtained for technical reasons, the animals were also scored 1. The investigators performing the injections and monitoring the mice were blinded towards the treatment groups. The statistical significance of differences between thrombus scores was tested using the the Kruskal-Wallis or Mann-Whitney U test.

The presence of a vaginal mucus plug in at least 2 females within the follow-up period for each these males treated with antibody or saline, confirmed actual sexual activity of the males.

KRIX-1, CHO-rec-KRIX-1 were able to prevent priapism in all mice tested ($p < 0.05$ versus saline) (Figure 7). In the group injected with 2 x 100 μg KRIX-1 antibody, none of the five males developed priapism; four of them were also free of thrombosis upon visual inspection and by microscopic analysis at the end of the experiment; the remaining male

did not show macroscopic thrombosis. For technical reasons, no histological analysis could be performed and the animal was therefore scored 1 (Figure 7), the maximal score which could have been attributed if the analysis had been performed.

A similar outcome was observed for the recombinant CHO-rec-KRIX-1 antibody: none of seven treated males developed priapism; five males were also free of macroscopic or microscopic thrombosis (Figure 7); one male showed only microscopically detectable thrombosis (score 1) (Figure 7) and one male was free of macroscopically visible thrombosis but could not be analyzed by microscopy and was therefore also scored 1 (Fig 3).

Example 6. Production and characterisation of variant of CHO-recKRIX-1 devoid of N-glycosylation site in the antigen binding site

Asn47Gln CHOrecKrix-1was produced by site directed mutagenesis on the pCR4-Blunt-TOPO-LE2E9H plasmid resulting in a single amino acid change in the heavy chain altering the Asn47 into Gln47 in order to disrupt the N-linked glycosylation site at Asn47-Thr49. Other plasmids comprising the coding sequence of the krix-1 antibody can similarly be used in the context of the present invention. Sequences comprising the CDRs of the heavy and light chains of Krix-1 are provided in SEQ ID NO:1 and SEQ ID NO: 2 respectively.

The mutagenesis at Asn 47 was obtained using the Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in combination with the following specific PCR primers:

Forward primer:

5'-CCTGCAAGACCTCTGGATAC***c***A***a***TTCACCGGCTACTCTGCTTCTGG-3' (SEQ ID NO: 7) corresponding to nt 119 to 164 of the Krix-1 Heavy chain sequence (capital) containing two altered nucleotides (a to c and c to a; bold italic);

Reverse primer:

5'-CCAGAAGCAGAGTAGCCGGTGAA***t***T***g***GTATCCAGAGGTCTTGCAGG-3' (SEQ ID NO: 8) corresponding to nt 119 to 164 of the LE2E9 Heavy chain sequence (capital) containing two altered nucleotides (g to t and t to g; bold italic)

Thr49Ala CHO-recKrix-1was produced by site directed mutagenesis resulting in a single amino acid change altering the Thr49 into Ala49 in order to disrupt the N-linked glycosylation site at Asn47-Thr49

This was obtained using the Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in combination with the following specific PCR primers:

Forward primer: 5'-CCTCTGGATACAACTTC***g***C***t***GGCTACTCTGCTTCTGG-3' (SEQ ID NO: 9) corresponding to nt 128 to 164 of the LE2E9 Heavy chain sequence (capital) containing two altered nucleotides (a to g and c to t; bold italic);

Reverse primer: 5'-CCAGAAGCAGAGTAGCC***a***G***c***GAAGTTGTATCCAGAGG-3' (SEQ ID NO: 10) corresponding to nt 128 to 164 of the KRIX-1 Heavy chain sequence (capital) containing two altered nucleotides (g to a and t to c; bold italic);

Asn47Glu CHO-recKrix-1was produced by site directed mutagenesis resulting in a single amino acid change altering the Asn47 into Glu47 in order to disrupt the N-linked glycosylation site at Asn47-Thr49

Forward primer:

5'-CCTGCAAGACCTCTGGATAC***g***A***g***TTCACCGGCTACTCTGCTTCTGG-3' (SEQ ID NO: 11) corresponding to nt 119 to 164 of the Krix-1 Heavy chain sequence (capital) containing two altered nucleotides (a to g and c to g; bold italic);

Reverse primer:

5'-CCAGAAGCAGAGTAGCCGGTGAA***c***T***c***GTATCCAGAGGTCTTGCAGG-3' (SEQ ID NO: 12) corresponding to nt 119 to 164 of the Krix-1 Heavy chain sequence (capital) containing two altered nucleotides (g to c and t to c; bold italic).

Asn47Asp CHO-recKrix-1was produced by site directed mutagenesis resulting in a single amino acid change altering the Asn47 into Asp47 in order to disrupt the N-linked glycosylation site at Asn47-Thr49.

Forward primer:

5'-CCTGCAAGACCTCTGGATAC***g***ACTTCACCGGCTACTCTGCTTCTGG-3' (SEQ ID NO: 13) corresponding to nt 119 to 164 of the Krix-1 Heavy chain sequence (capital) containing one altered nucleotide (a to g; bold italic);

Reverse primer:

5'-CCAGAAGCAGAGTAGCCGGTGAAGT***c***GTATCCAGAGGTCTTGCAGG-3'

(SEQ ID NO: 14) corresponding to nt 119 to 164 of the Krix-1 Heavy chain sequence (capital) containing one altered nucleotide (t to c; bold italic)

After sequence verification, the mutated heavy and wild-type (native) Krix-1 light chain were cloned into the pEE6.4 and pEE14.4 vector (Lonza Biologics, Portsmouth, NH) respectively. The two vectors were combined to a double gene vector – containing both heavy and light chain - using the *Not*I and *Sal*I restriction sites present in both vectors. Heavy and light chain expression in eukaryotic cells is under the control of the hCMV-MIE promoter (present in pEE14.4 and pEE6.4). The double gene vector was linearised with *Sal*I before transfection.

The linearised vector was used for stable transfection of CKO-K1 cells using the FuGENE6 transfection reagent (Roche, Brussels, Belgium) according to the manufacturer's instructions. The transfected cells were cultured in glutamine-free DMEM (JRH Biosciences, Lenexa, KS) supplemented with FBS 10%, GS Supplement (JRH Biosciences, Lenexa, KS) and 25µM L-Methionine Sulfoximine (MSX) (Sigma-Aldrich, Bornem, Belgium) for selection.

The best producers were adapted to growth in serum-free medium (EX-CELL 302 serum-free medium w/o L-Glutamine, JRH Biosciences, Lenexa, KS) - supplemented with 25µM MSX and GS Supplement - by step-wise reduction of the FBS to 0%. The best expressing (ELISA with anti-humanIgG4 detection antibody) functional cell line was used for batch production of the mutated rec-mAb-Krix-1, either using the adherent or the suspension cell line.

The recombinant antibody was purified from the cell culture supernatant by affinity chromatography using a HiTrap rProtein A FF column (Amersham Biosciences, Uppsala, Sweden). After concentration the rec-mAb-LE2E9Q (A, E and D resp.) were assayed for functionality (Chromogenic assay to evaluate the ability of the mutated rec-mAb Krix-1 to inhibit fVIII activity). Inhibitory capacity towards fVIII was compared to that of the wild type rec-mAb Krix-1.

Measurement of surface plasmon resonance (SPR).

The rate of FVIII association and dissociation to Gln47Glu, Thr49Ala and native CHO-rec-KRIX-1 was analysed using a Pharmacia Biosensor BIAcore™ instrument (Pharmacia Biosensor AB). Purified antibody (20 µg/ml in 10 mM sodium acetate buffer pH 5.0) was immobilised on the activated surface of a CM5 sensor chip, according to the manufacturer's instructions. All binding experiments were carried out in HBS at a constant flow rate of 10 µl/min. FVIII in Hepes Buffered Saline (HBS) was infused at various concentrations over the antibody coated on the sensor chip surface. At the end of each cycle, the surface was regenerated by flushing HCl, pH 2, for 36 sec. Control experiments ensured that FVIII bound only to insolubilised antibody. Thus, rFVIII did not bind to the sensor chip in the absence of antibody, and preincubation of rFVIII with soluble antibody prior to addition to the chip completely prevented FVIII binding.

Association and dissociation rate constants were determined by non-linear fitting of individual sensorgram data (39) using the BIA evaluation 2.1 software (Pharmacia Biotech, Uppsala, Sweden). Values of k_{ass} and k_{diss} were determined by averaging the values obtained for individual curves established with various analyte concentrations. Values of k_{diss} were determined from the individual curves obtained with only the highest analyte concentration, in order to reduce bias due to rebinding of the analyte to free immobilized ligand. All data were analysed after correction of the baseline by subracting the response observed before injection of the analyte (rfVIII) from the response values obtained during the association and dissociation phases.

The dissociation constant (K_D) of FVIII from Gln47Glu, Thr49Ala and native CHO-rec-KRIX-1 was very similar (Table 1). Accordingly, the glycosylation site in the antigen binding site of mAb Krix-1 influences the antibody inhibitory activity but does not contribute significantly to binding to FVIII.

Table 1. surface plasmon resonance analysis of FVIII binding to mAb Krix-1 and derivative thereof.

K_D (nM)

Modified mAb Krix-1 (LCL):

CHO-recKRIX-1	0,14 ± 0,03
Gln47Glu-CHO-recKRIX-1	0,17 ± 0,02
Thr49Ala-CHO-recKRIX-1	0,13 ± 0,01

Example 7. Prevention of arterial and venous thrombosis in baboons.

Methods

Protocol

Male baboons (Papio ursinus) were used. The animals weighed between 8 and 17 kg and were disease-free for at least 6 months prior to the experiments. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa.

Permanent polytetrafluoroethylene (Teflon) and silicone rubber (Silastic) arteriovenous (AV) shunts were implanted in the baboon femoral vessels. Blood flow through the shunts varied between 100 and 120 mL/min. Handling of the baboons was achieved through anesthesia with ketamine hydrochloride (Anaket-V, Centaur Laboratory).

In each experiment, a thrombogenic device prefilled with saline to avoid a blood-air interface was incorporated as an extension segment into the permanent arteriovenous shunt by means of Teflon connectors (Kotze et al., 1993). Platelet-dependent arterial thrombus was induced by using Dacron inserted into the wall of Silastic tubing (3-mm inside diameter) according to Hanson et al., 1985 (Figure 10).

The Dacron vascular graft material (1.26 cm^2) served as a generator of platelet-dependent arterial-type thrombosis. An expansion chamber (3.77 cm^2) was used to generate coagulation-dependent venous thrombosis. Blood flowed through the

thrombogenic devices at a rate of approximately 120 ml/min. The initial shear stress was 318 sec^{-1} for the Dacron section and 10 sec^{-1} for the expansion chamber.

In the control studies, the devices were kept in place for 60 minutes or until they occluded, after which they were removed and blood flow through the permanent AV-shunt re-established. The baboons were then treated with a single intravenous bolus of 1.25, 2.5 or 5 mg/kg LCL- or PerC6 Krix-1. The thrombogenic devices were placed for 60 minutes 1h after antibody injection, after which the devices were removed and blood flow through the permanent AV-shunt reestablished. Additional 60-minute studies were carried at 24h and 48h after the antibody bolus injection. The extracorporeal shunts were then removed after the last thrombosis experiment. Blood samples were taken according to the sampling schedule either directly from the shunt or by venopuncture. FVIII activity, mAb Krix-1 concentrations monitored, PT, APTT, fibrinogen, were measured on all samples.

Graft imaging.

Autologous platelets were labeled with 111In-tropolone and reinjected into the animal 1h before the start of the control experiment. This allowed image acquisition on day 0, 1 and 2. To provide image acquisition on day 6 or 14 the labeling procedure was repeated. Image acquisition of the grafts was done with a gamma scintillation camera fitted with a high-resolution collimator. The images were stored on and analysed with a computer imaging and analysis system interfaced with the scintillation camera. Dynamic image acquisition, 3-minute image of a 5ml autologous blood sample were also acquired each time the grafts were imaged to determine blood radioactivity (blood standard). Regions of interest of the graft and expansion segments were selected to determine the deposited and circulation radio-activity in the dynamic image. The total number of platelets deposited on the vascular graft material and in the expansion chamber were calculated.

In 6 animals treated with Asn47Glu, platelet deposition was lower than in the control animal treated with saline in both the venous and arterial thrombosis chambers (Figure 11).

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CLAIMS

1. A method for obtaining a library of at least two modified antibodies with variable maximal inhibitory activity with substantially unaffected affinity, said method comprising modifying the glycosylation in the variable region of an inhibitory antibody, and selecting those antibodies for which affinity is not substantially affected

2. The method of claim 1, wherein said inhibitory antibody is an antibody inhibiting the blood coagulation cascade.

3. The method of claim 1, wherein said inhibitory antibody is an antibody inhibiting FVIII.

4. A library of modified anticoagulant antibodies obtained by the method of any of claims 1 to 3.

5. The method of any of claims 1 to 4, wherein said inhibitory antibody is Krix-1.

6. An anticoagulant antibody derived from Krix-1, characterized in that one or more modifations in the glycosylation of its variable region result in a modified inhibitory activity without significantly affecting the affinity of the monoclonal antibody to FVIII.

7. The anticoagulant antibody of claim 6, wherein said modification in the glycosylation of its variable region is a modified N-glycosylation.

8. The anticoagulant antibody of claim 6, wherein said modified inhibitory activity is results in an inhibition of between 20 and 95% of FVIII activity and ensures prevention of thrombosis in mammal models of thrombosis.

9. The anticoagulant antibody of any one of claims 6 to 8, which is LE2E9Q or LE2E9A or an Fab fragment or F(ab')2 fragment of the monoclonal antibody LE2E9Q or LE2E9A.

10. The anticoagulant antibody of any one of claims 6 to 8, wherein said modified glycosylation is obtained by site-directed mutagenesis to introduce a glycosylation site in the variable region of the antibody.

11. The anticoagulant antibody of any one of claims 6 to 8, comprising an immunoglobulin heavy chain comprising a sequence having at least 90% sequence identity to SEQ ID Nos 1, 2, 3, or 4.

12. A mixture of two or more antibodies antibodies according to claims 6-8 mixed together in an appropriate ratio to achieve a given maximal inhibition of FVIII activity, whatever the excess of the mixture of antibodies over FVIII.

13. A pharmaceutical composition comprising the antibodies of any one of claims 6 to 11 or the mixture of claim 12.

14. A method of treatment comprising administering an effective dose of a therapeutic monoclonal antibody or fragment thereof modified in such a way as to modify or introduce a glycosylation site in the antigen binding site of the antibody in order to modify the inhibitory effect of the said antibody on the interaction(s) of the ligand(s) recognized by the said antibody with other proteins or reagents interacting with the said ligand.

15. A method for treatment and prevention of thromboembolic disorders (including, prevention of deep vein thrombosis and pulmonary embolism secondary to surgical intervention, immobilization or chronic hereditary or acquired thrombophilia, and treatment of deep vein thrombosis, pulmonary embolism, stroke, atrial fibrillation, and non Q wave myocardial infarct, non ST elevated myocardial infarct or unstable angina), comprising administering an effective dose of a monoclonal antibody or fragment thereof obtained by the method of claim 1.

16. A method for treatment and prevention of thromboembolic disorders comprising administering an effective dose of a monoclonal antibody or fragment thereof, according to any one of claims 6 to 11, the mixture according to claim 12 or an antibody obtained according to the method claim 1 and administered concomitantly to drug(s) inhibiting platelet aggregation, such as aspirin.

17. A method for treatment of acute myocardial infarct comprising administering an effective dose of a monoclonal antibody or fragment thereof according to any one of claims 6 to 11, the mixture according to claim 12 or an antibody obtained according to the method claim 1, and administered concomitantly to drug(s) inhibiting platelet aggregation, such as abciximab (Rheopro^R) or antithrombolytic agents (including tissue plasminogen activator, staphylokinase or microplasmin).

18. The method according to claim 15 or 16, wherein said monoclonal antibody is an anticoagulant monoclonal antibody derived from Krix-1 and carrying a mutation in the N-glycosylation site of the antigen binding site.

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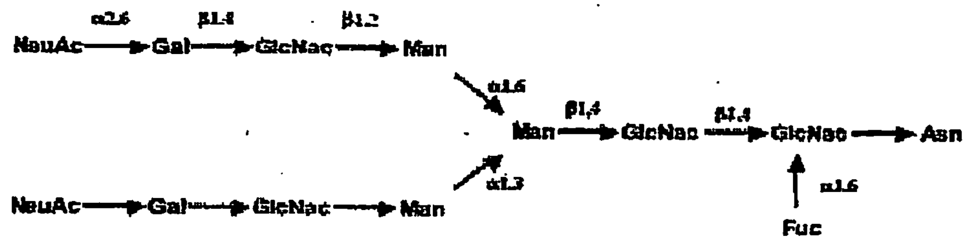
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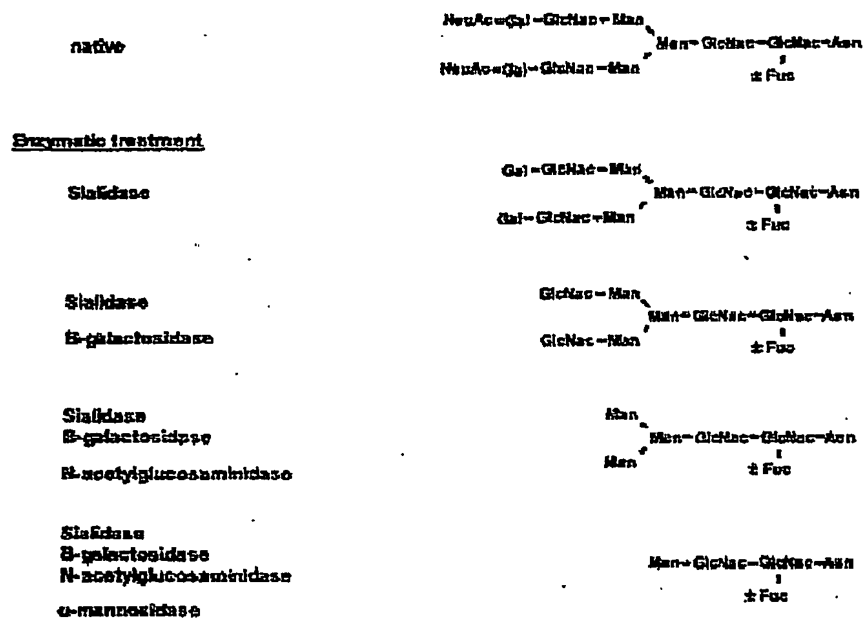
Figure 1


native
NeuAc→Gal→GlcNac→Man
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Man→GlcNac→GlcNac→Asn
± Fuc
Enzymatic treatment
Sialidase
Gal→GlcNac→Man
Gal→GlcNac→Man
Man→GlcNac→GlcNac→Asn
± Fuc
Sialidase
ß-galactosidase
GlcNac→Man
GlcNac→Man
Man→GlcNac→GlcNac→Asn
± Fuc
Sialidase
ß-galactosidase
N-acetylglucosaminidase
Man
Man
Man→GlcNac→GlcNac→Asn
± Fuc
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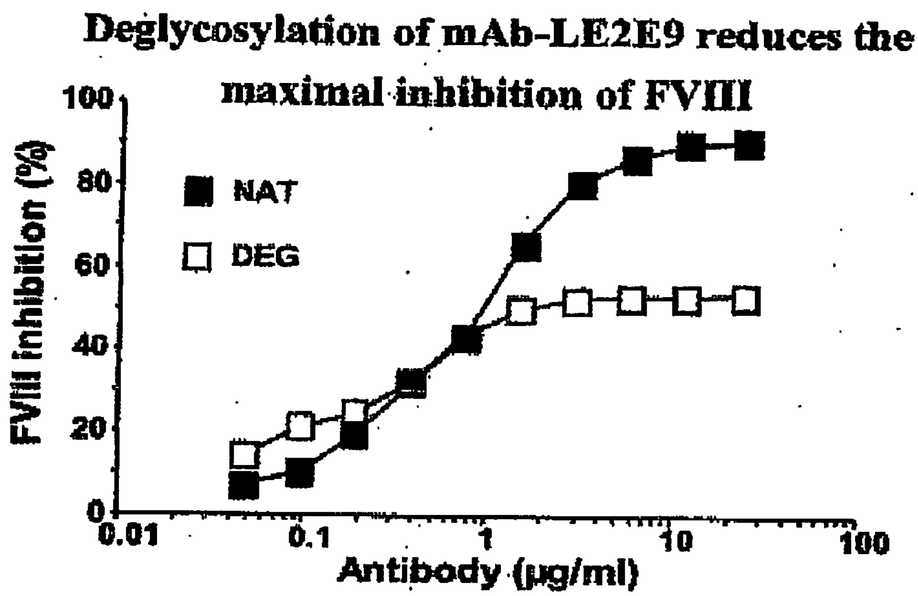
Man→GlcNac→GlcNac→Asn
± Fuc

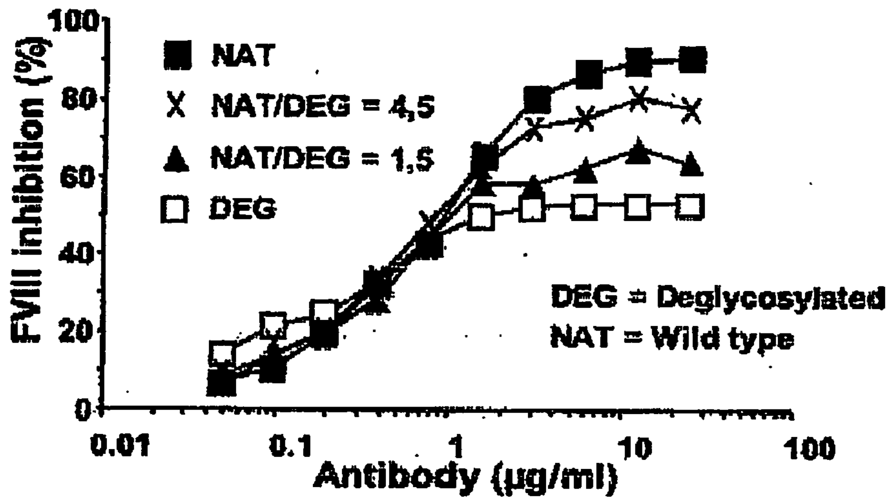
Figure 2.



DEG = Deglycosylated KRIX-1 NAT = Native KRIX-1

Figure 3.

Mixing deglycosylated mAb-LE2E9 with wild-type mAb-LE2E9 reduces the maximal inhibition of FVIII



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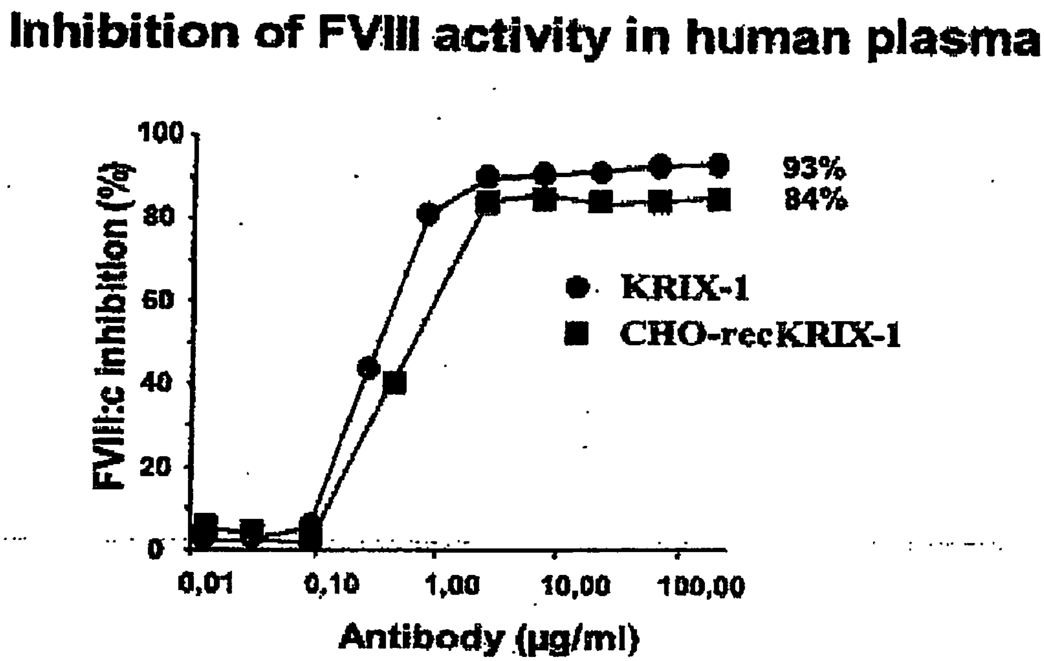
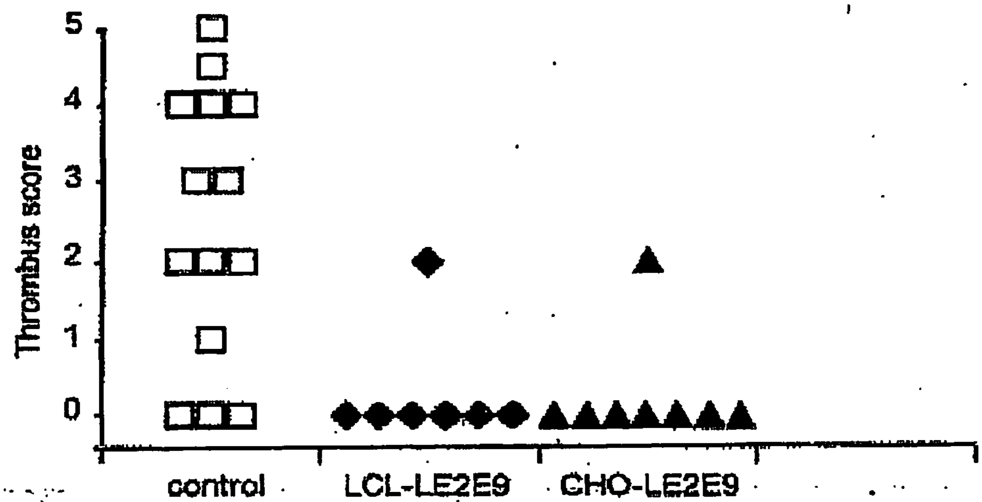

Inhibition of FVIII activity in human plasma
100
80
60
40
20
0
FVIII:c inhibition (%)
0,01
0,10
1,00
10,00
100,00
Antibody (µg/ml)
93%
84%
KRIX-1
CHO-recKRIX-1

Figure 5.



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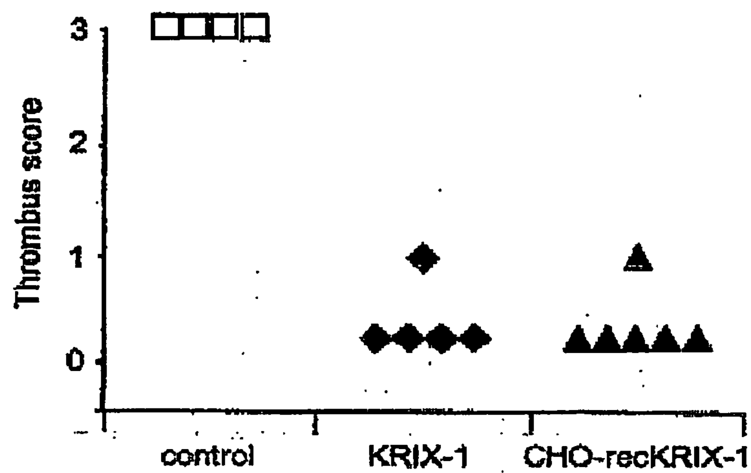

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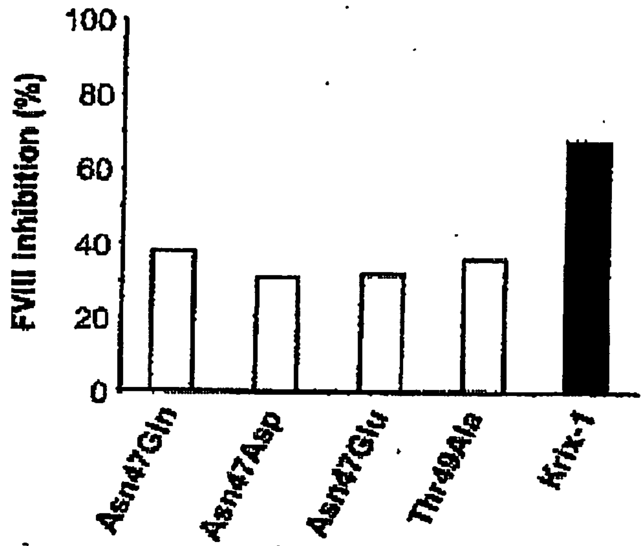
100
80
60
40
20
0
FVIII inhibition (%)
Asn47Gln
Asn47Asp
Asn47Glu
Thr49Ala
Krix-1

Figure 8.

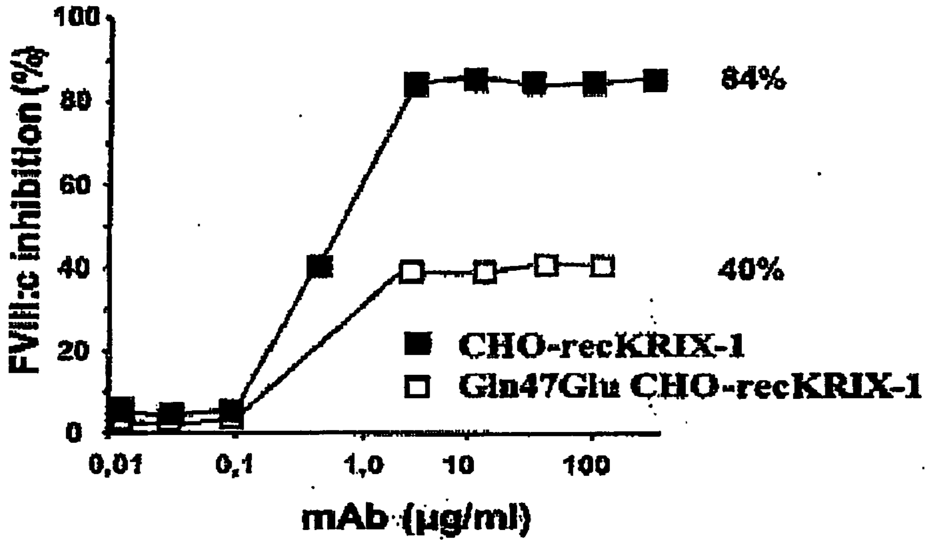
100
80
60
40
20
0
FVIII:c inhibition (%)
84%
40%
CHO-recKRIX-1
Gln47Glu CHO-recKRIX-1
0,01
0,1
1,0
10
100
mAb (µg/ml)

Figure 9.

Schematic representation of the venous thrombosis chamber

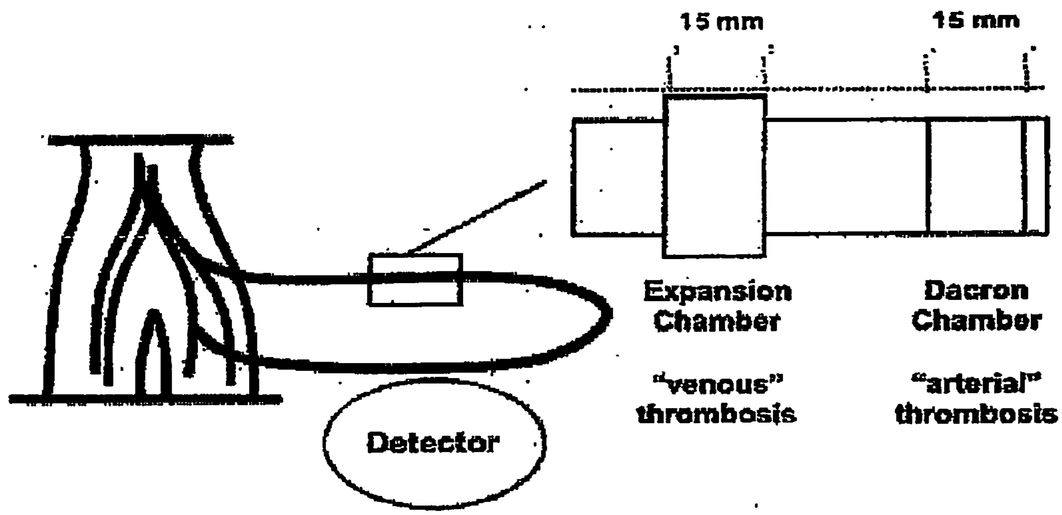

Figure 10

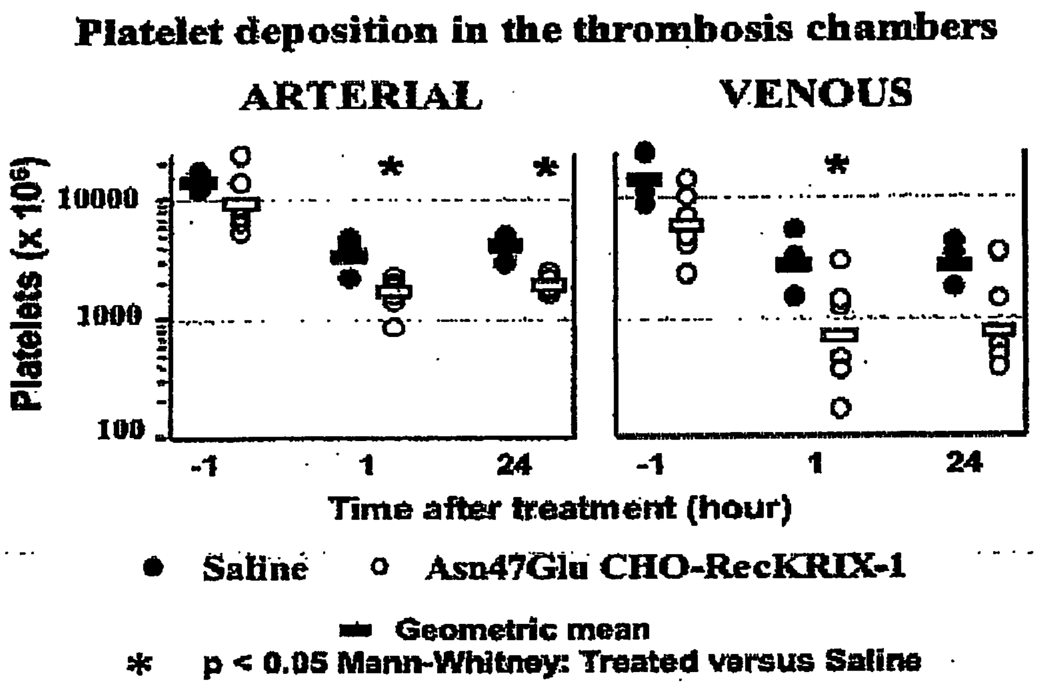
Platelet deposition in the thrombosis chambers
ARTERIAL
VENOUS
Platelets (x 10^6)
10000
1000
100
-1
1
24
-1
1
24
Time after treatment (hour)
Saline
Asn47Glu CHO-RecKRIX-1
Geometric mean
p < 0.05 Mann-Whitney: Treated versus Saline

Figure 11

PCT/BE2004/000118